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CONTENTS

No. 1   JANUARY, 1940

No. 2   FEBRUARY, 1940

iii

No. 3 MARCH, 1940

No. 4 APRIL, 1940

No. 5 MAY, 1940

No. 6   JUNE, 1940

SIMPLIFIED RATIONS FOR THE CHICK [1]

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FOUR FIGURES

Sometime ago Hogan and Boucher ('33) devised a simplified ration for the chick which contained all of the vitamins in soluble form. This ration supported a normal rate of growth, and four generations of chicks, which received no other food, were reared successfully (Hogan, Boucher and Kempster, '35). This ration gave every evidence of adequacy during a period of over 2 years and then inexplicable failures were encountered and it became necessary to start anew.

EXPERIMENTAL

The experimental procedure is the same as was used before. Day-old single comb White Leghorn chicks, obtained from the College Poultry Department have been used exclusively. These were kept for 3 weeks in electrically-heated battery brooders, provided with screen floors, and then transferred to a larger fattening brooder, also provided with screen floors. The temperature of the room was regulated automatically during the cooler months, but there was no provision for cooling the room during the hot summer weather. The rations were of the simplified type and are described below.

Simplified rations for chicks

	%		%
Casein [2]	35	Cod liver oil	2
Cellulose	3	Soy bean oil	8
Salts [3]	4	Vitamin carriers	..
Lard	7	Starch to make 100	..

[1] Contribution from the Missouri Agricultural Experiment Station Journal Series No. 622.

[2] Extracted with dilute acetic acid.

[3] Mixture 351 of Hubbell, Mendel and Wakeman ('37).

1

The amounts of the vitamin carriers as given later refer to their dry matter content, and when included in rations they displaced an equal weight of starch. During the early stages of this study each chick received 40 γ of thiamin, and 20 γ of flavin daily by pipette. More recently these vitamins were included in the ration at a level of 200 γ of thiamin and 400 γ of flavin per 100 gm. of ration. The crystalline vitamins were added to the food at intervals of 2 or 3 days.

The key to such success as had been attained by Hogan and Boucher lay in the use of liver extracts, for we have never been able to obtain consistently, in soluble form, from any other source some of the vitamins required by the chick. It was suspected therefore that the later failures mentioned were due to variability in the liver extracts, so in future studies it was decided to rely on extracts prepared in the laboratory. Up to this point the extracts, two in number, had been obtained from commercial sources. A water extract of liver is the starting material in their preparation and this is fractionated with alcohol. One fraction is soluble, the other insoluble in 70% alcohol. Of these two, the soluble fraction seemed the most useful for our purpose, but with some shipments of the commercial products the performance of the chicks was inferior unless both fractions were present in the ration. This indicated that the activity of the extracts was due to more than one component, so the procedure was modified in an attempt to devise a method that would give a more complete separation of the active agents. This attempt has not been entirely successful, but after the change in procedure became effective, the immediate difficulties due to growth failure disappeared. A condensed description of the procedure appears in table 1, and the yields from two representative shipments are reported in table 2.

As a standard of success in our feeding trials two growth curves have been used. One is that published by Heuser and Andrews ('32), and selected because it was the most rapid we have seen for chicks subsisting on practical rations. The other was prepared from our own data for chicks reared on a ration

of natural foodstuffs. In each feeding trial we always run such a control group. This is used as a control to be sure that failures are due to inadequacy of the rations and not to subnormal chicks. As is evident from the graphs, the growth rate on our practical ration is a severe criterion of performance.

TABLE 1

Plan followed in the preparation of the liver extracts used

Preparation of liver extracts: 50 pounds fresh beef liver dried at 55°–60°C. Extracted five times with 9 liters of 95% ethyl alcohol at 70°C. each time

Alcohol extract. Alcohol removed in vacuo, lipoids separated by gravity and by extraction with ether

Residue. Extract five times with 9 liters of water at 70°C. for each extraction

Fat free alcohol extract (3849), adsorb with fuller's earth

Lipoids, discarded

Water extract, water removed in vacuo (3776)

Residue (3777) hydrolyze with H_2SO_4

Hydrolysate (3846)

Fuller's earth adsorbate (3774)

Filtrate (3775)

TABLE 2

Yields of liver fractions

	FRESH LIVER	DRY LIVER.	LABORATORY NO. OF FRACTIONS			
			3774	3775	3776	3777
	gm.	gm.	gm.[1]	gm.	gm.	gm.
A	329	100	1.6	2.5	6.0	55.5
B	332	100	2.0	3.0	7.0	63.1

[1] The weights as given are of the fuller's earth adsorbate. Only a small fraction was derived from the liver extract.

Adequacy of liver fractions. Figure 1 summarizes the rates of growth observed in some of the individual feeding trials. Their reliability has been established, in some cases, by frequent repetition.

Ration 3992 contains both the alcoholic extract and the water extract, and it will be observed that the rate of growth is practically identical with that of the control group. There was one mortality in this group, but it was the only one of the eighteen chicks that received this ration.

As is indicated in table 1, the alcohol soluble material was fractionated by adsorption on fuller's earth, so this adsorbate and the filtrate were combined in the rations of a number of

Fig. 1   The liver fractions here studied contained all of the unrecognized vitamins required by the chick. Rations 3874 and 3992 contained liver extracts only and permitted chicks to grow at the normal rate. The curves for ration 3982 show that in rations of this type 4% of the water-soluble fraction is not enough.

Ration 3875 contained the liver residue, and supported a remarkable rate of growth. The hydrolyzed liver residue, present in ration 3932, was of some value but less effective than the residue itself.

groups. No. 3774 is the fuller's earth adsorbate of the alcohol soluble fraction, no. 3775 is the filtrate, and no. 3776 is the water extract.

When the three soluble fractions were included at the levels shown in ration 3874 the rate of growth was excellent, but when the amounts of preparations 3775 and 3776 were reduced,

as in ration 3982, the response was less satisfactory. In ration 3875 the amounts of the soluble fractions were still further reduced but the thoroughly washed liver residue was included in addition. It is very evident that the liver residue still contained one or more substances which accelerates the rate of growth in a very significant manner. Ration 3932 is very similar to ration 3875, but the acid hydrolyzed liver residue has been substituted for the liver residue. The rate of growth was less rapid, but was still satisfactory when compared with any conventional growth standard. In figure 3 is presented further evidence of the value of the hydrolyzed material.

It is evident that the liver fractions are a satisfactory source of the unrecognized vitamins required by the chick. If supplied in sufficient amount the fractions soluble in alcohol and in water are adequate by themselves. If the amounts of these extracts are much reduced, then it is necessary to include the hydrolyzed liver residue in addition, in order to obtain a normal growth rate.

Distribution of the required vitamins. The rations described thus far contained both the alcohol and water soluble fractions. One may ask, therefore, whether both are essential, or helpful. Illustrations of our data on that point are presented in figure 2.

Rations 3881, 3876, and 3877 contained varying amounts of the water soluble fraction, no. 3776, but none of the alcohol soluble fraction. In each instance the growth rate was subnormal. Furthermore the rate of growth was the same, whether the ration contained 10 or 16% of this supplement. Reducing the amount from 10 to 6% of the ration did not greatly reduce the adequacy of the ration. This shows that the water soluble fraction must be supplemented with the alcohol soluble fraction.

Some attention has also been given to the adequacy of the alcohol soluble fraction alone, illustrated by ration 3789 which also contained the liver residue. The growth rate was slow and the mortality was 100%. In ration 3788 the liver residue was omitted and 2% of the water extract was included. The

rate of growth was still below the optimum on this low level, but it is evident that in this type of ration the water extract is indispensable. The essential nature of this component is brought out still more clearly in ration 3887, which supports an extremely rapid rate of growth. Thus ration 3789, a complete failure, was converted into a decided success merely by adding to it 2% of the water soluble fraction.
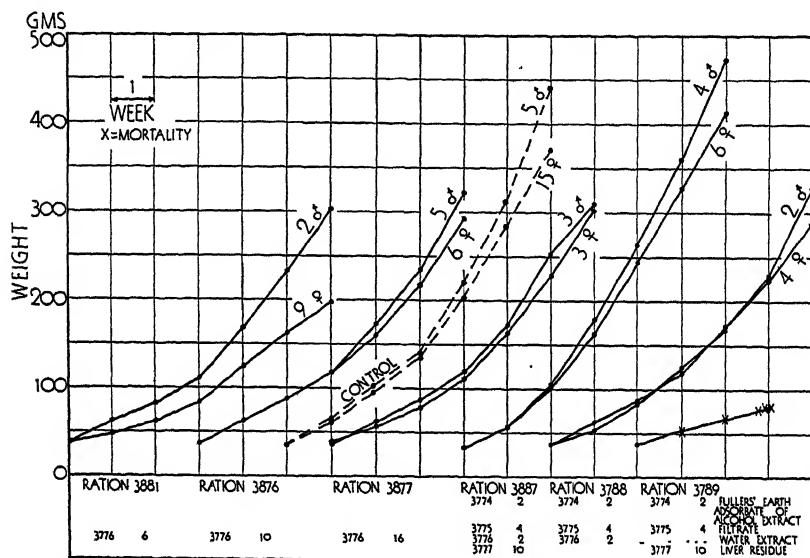
Fig. 2   Rations 3876, 3877, and 3881 contain the water-soluble fraction only, and are unsatisfactory. Ration 3789 contains the alcohol-soluble fraction only and gives a complete failure. Ration 3887 contains both soluble fractions, and is a marked success.

It may be concluded, therefore, that the alcohol extract, although not absolutely indispensable in rations of this type, yet is required in order to secure a high degree of success. The water extract is indispensable, but by itself is inadequate. Presumably a more refined method of preparation should give a sharper separation, and then both the alcohol and water soluble fractions would be found to be absolutely indispensable.

Nutritional properties of the hydrolyzed liver residue. How important is the hydrolyzed liver residue in formulating a simplified ration? Figure 3 is presented to show that the acid hydrolyzed liver may contribute something of value, although, as was brought out previously, it is not essential if the other soluble fractions are included in sufficiently large amounts.

Ration 3993 does not contain acid hydrolyzed liver, and chicks which received it grew slowly. When the hydrolyzed product was included, as in ration 3981, the growth rate was markedly accelerated. When, as a further addition, the alcohol soluble fractions were also included (ration 3980), a very
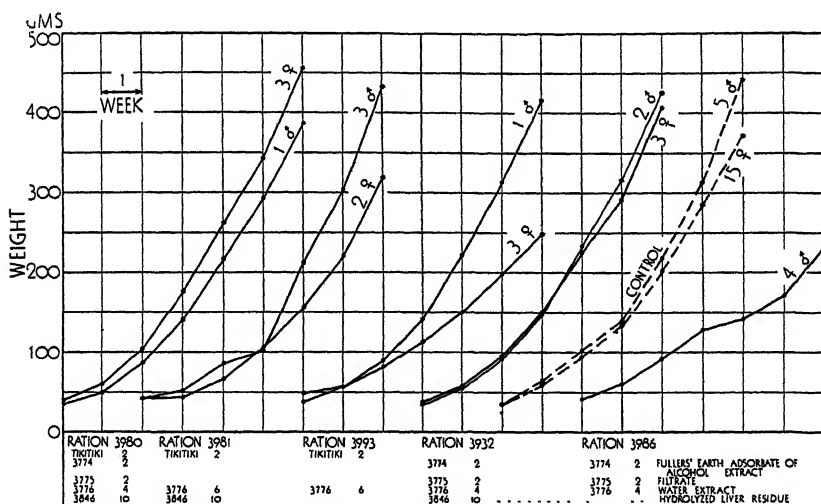
Fig. 3 Rations which lacked the hydrolyzed residue and contained small amounts of the soluble fractions, such as 3986 and 3993, were unsatisfactory. When the residue was included, as in rations 3980 and 3932, the rate of growth was normal.

considerable acceleration of growth occurred again. Ration 3986 does not contain acid hydrolyzed liver and is altogether unsatisfactory. When acid hydrolyzed liver was included as in ration 3932 the rate of growth surpasses those that have been described in the literature as normal.

It is concluded, then, that when the alcohol and water extract are supplied at a low level, the ration can be made com-

plete by including the hydrolyzed liver residue. If, however, the alcohol and water extracts are supplied at a sufficiently high level, then the hydrolyzed liver residue does not effect any marked further improvement.

The nature of the active agent present in hydrolyzed liver deserves some comment. We have tentatively assumed it to be a vitamin. However, Arnold, Kline, Elvehjem and Hart ('36) demonstrated that the growth rate of chicks can be definitely improved by the addition of arginine to a natural ration in which corn and wheat proteins are fortified with casein or when casein is the principal protein. Klose, Stokstad and Almquist ('38) reported that if the ration contained only 20% of casein the addition of arginine improved the growth rate, but if the ration contained 30% of casein, then arginine reduced the growth rate if it had any effect at all. Our rations contain 35% of casein so presumably the activity of the acid hydrolyzed product was not due to arginine. However, that possibility was explored by adding 1% of arginine carbonate to rations that did not contain acid hydrolyzed liver. The rate of growth was not at all accelerated, from which we may presume that the active agent in hydrolyzed liver is not arginine. Our data do not enable one to decide whether it is an amino acid, or some other substance liberated by hydrolysis.

The usefulness of tikitiki. May tikitiki make any important contribution to a simplified ration for chicks? This material was prepared by the method of Wells ('21). As shown in the previous figures, we have frequently used tikitiki as a carrier of soluble vitamins. The curves presented in figure 4 show that it contains a nutrient which is required by the chick.

Ration 3944 does not contain tikitiki, and is a failure. Ration 3931 is otherwise similar to ration 3944, but it contains 2% of tikitiki and is much superior. Ration 3986 also does not contain tikitiki and, practically speaking, it is a failure. When tikitiki is included though, as in ration 3964, the rate of growth is almost normal. Ration 3932 contains all of the liver fractions and no tikitiki, and is a complete success, which indicates that the alcohol soluble fraction contains the same factors as

does tikitiki. This is shown still more clearly in ration 3980 which contains 2% of tikitiki. However, chicks do not grow more rapidly on this ration than on ration 3932 which contains no tikitiki. It may be concluded then, that if the alcohol soluble fraction is present in liberal amounts, tikitiki may be omitted. Usually, however, chicks grow more rapidly when it is included.
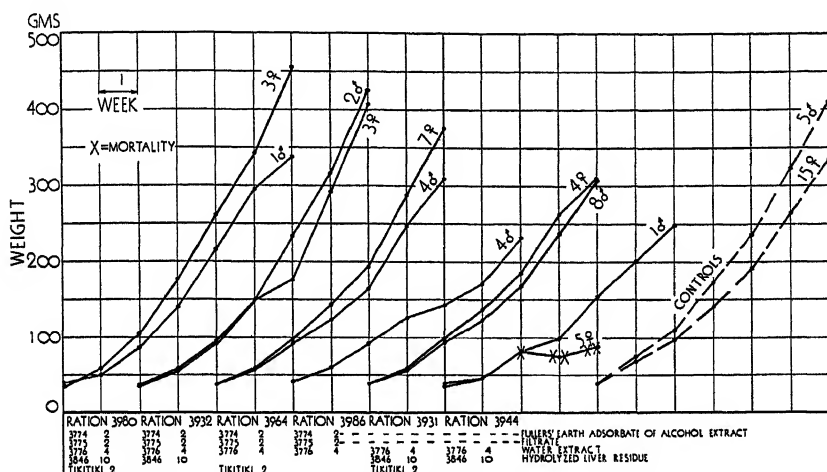
Fig. 4   Ration 3944 does not contain tikitiki and is a failure. The addition of tikitiki, as in ration 3931, brings about marked improvement. Ration 3986 lacks tikitiki and is unsatisfactory, but when tikitiki is included as in ration 3964, the growth rate is greatly accelerated. Ration 3932 contains the alcoholic extract and it is not improved when further fortified with tikitiki, as in ration 3980.

The mortality rate is highly significant in an assay of the adequacy of a ration, and therefore a record of the deaths is summarized in table 3. Only the chicks on rations that offered some promise were considered in the preparation of this table, although it should be mentioned that some of the rations are not described in this report. The chicks used for these calculations are distributed among three groups, depending on whether their ration contained the liver residue, the hydrolyzed liver residue, or neither of these constituents.

None of the chicks was obviously defective when placed under observation, although some were smaller and weaker

than others. Furthermore, there was no weeding out after the feeding trials started, so the total mortality was satisfactorily low under the circumstances. A total of 290 chicks is included in table 3, and only three, slightly over 1%, died after they were 2 weeks old. It is evident, then, that there are no unusual difficulties in rearing chicks on these simplified rations.

TABLE 3

Mortality rate

RATION CONTAINS	NUMBER OF CHICKS	TOTAL MORTALITIES	DEATHS DURING		
			1st week	2nd week	3rd–6th week
		%	%	%	%
Liver residue	114	3.5	0	1.75	1.75
Hydrolyzed liver residue	79	8.8	3.5	5.3	0
Soluble extractives only	97	6.2	4.1	1.1	1.0

SUMMARY

1. If recognized vitamins, i.e., A, B_1, B_2 and D, are otherwise provided, then all the unrecognized vitamins required by the chick are present in alcohol and water extracts of liver.

2. Partial separation of the unidentified essential nutrients can be attained by extracting first with alcohol and then with water. Both extracts must be present if the ration is to be adequate.

3. When the alcohol and water extracts were supplied at a low level, then the liver residue, or hydrolyzed liver residue, supplied some essential nutrient. If, however, the extracts were supplied at a sufficiently high level, then there was no further increase in the effectiveness of the ration when the hydrolyzed product was included.

4. Tikitiki contains an essential nutrient, but the data thus far available indicate that this same nutrient is present in the alcohol soluble fraction, and presumably in even greater concentration.

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ADEQUACY OF A MILK DIET FOR THE RAT [1]

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The major nutritional deficiencies of milk are well known, but even after these are supplied it is still questionable whether this food, as the sole article of diet over long periods of time, will maintain the optimum nutritional state. Of the various investigators who have attacked this problem, Daniels and Everson ('35) were probably the most successful, and their paper should be consulted for a review of the earlier literature. These workers were primarily interested in another problem, however, and used too few animals, over periods that were too short for a precise assay of the adequacy of a food. The tests were conducted with rats and the third generation consisted of four females, all of which produced first litters and three produced second litters. The fourth generation consisted of five females and the experimental period ended with their first litters.

At about this same time an investigation was under way in this institution which required that female rats be maintained throughout the greater part of their reproductive periods on a milk diet. Since fluid milk is too dilute to permit lactating rats to consume the optimum amount of energy, it was fortified by the addition of whole milk powder. The number of litters produced was far below that expected, and when the original investigation was completed we took charge of the female progeny in an attempt to discover the nature of the deficiency.

13

EXPERIMENTAL

The initial animals used in this investigation had received from the time they were weaned a milk diet only, consisting of equal parts by weight of fresh unpasteurized milk and whole milk powder.[2] This was supplemented with 2 cc. of mineral mixture 2089 (table 1) per 100 gm. of the milk preparation. The average amount of the milk mixture consumed was 25 cc. per rat daily, and it was estimated that this amount of food contained 2.5 mg. of added iron, 0.254 mg. copper, 0.167 mg. manganese, and 0.076 mg. of iodine. The milk was from pure

TABLE 1
Composition of mineral mixtures

COMPOUND	WEIGHT		ELEMENT	WEIGHT OF ELEMENT PER RAT DAILY	
	2089 [1]	2089A [2]		2089	2089A
	gm.	*gm.*		*mg.*[3]	*mg.*
$FeSO_4 \cdot 7H_2O$	5.00	5.00	Fe	2.5	5.00
$CuSO_4 \cdot 5H_2O$	0.40	0.63	Cu	0.254	0.80
$MnSO_4 \cdot 4H_2O$	0.27	0.82	Mn	0.167	1.02
KI	0.04	0.04	I	0.076	0.15
HCl conc.	1.0 cc.	1.0 cc.	.		
H_2O	200.0 cc.	200.0 cc.			

[1] Two cubic centimeters per 100 gm. of ration.
[2] One cubic centimeter per rat per day.
[3] Estimated from daily food intake.

bred Holsteins. During the growing season the cows, except for the dry summer of 1936, had adequate pasture of lespedeza, sweet clover, or Sudan grass. No information on the previous litter records was available beyond the statement that few litters had been produced, at very irregular intervals.

The animals were maintained on this same ration for some weeks, but in conformity with their previous history their reproductive activity continued at a very low level. It was suggested that the limiting factor was one of the known vitamins, and the milk ration just described was changed to include the more commonly used vitamin carriers. This new ration contained 50 parts of fresh unpasteurized milk,

[2] Klim, obtained from the Borden Company, New York.

and 50 parts of a mixture made up of 88.5% of whole milk powder, 10% of dried yeast, 0.5% of cod liver oil, and 1.0% of wheat germ oil. The mineral supplement was unchanged. This modification did not improve the reproductive performance and after a time the inclusion of yeast, cod liver oil, and wheat germ oil was discontinued. These females were then discarded and the observations continued with their daughters, designated as the first generation. They were weaned when 28 days old and distributed in groups of three or four in metal cages provided with screen floors of $\frac{1}{2}$ inch mesh. Their daily intake of food, and of minerals (mixture 2089) was approximately the same as that of their mothers. The freshly mixed diet, in glass or porcelain containers, was supplied each morning and evening. These experimental periods began in December, 1934, and in the following January.

When the females had attained weights of 175 to 200 gm. they were mated with a male from the stock colony. They were weighed weekly and when they became pregnant, as shown by a marked gain in weight, were placed in individual cages. The young were weaned when they were 28 days old and the mothers returned to the breeding cage without a rest period. Females on the milk ration were not discarded until they had failed to produce a litter for an average of 90 days.

The controls were females in the stock colony which were maintained on Steenbock's stock diet ('23). Each of them received 25 cc. of milk daily except when lactating and then the amount was increased to 35 cc. When these weaned their litters they, too, were returned at once to the breeding cage, with no rest period. This no doubt accounts in large measure for the high infant mortality in both groups. These animals were discarded if they did not produce a litter within 60 to 70 days after they were returned to the breeding cage. These rats are not classified by generations, either in the text or in the figures. They and their ancestors had received this same ration for approximately 15 years, and over long periods of time the reproductive rate does not materially change.

In the meantime the report of Daniels and Everson ('35) appeared which suggested that their ration may have been superior to the one we were using. Since the milk diet they used contained considerably larger quantities of the added minerals our ration was changed in July, 1935. From that time on 1.0 cc. of mixture 2089A, described in table 1, was supplied to each animal daily. The mineral solution was placed in the food container each morning with a small amount of milk and when that was consumed, usually in an hour or 2, the remainder of the allowance of milk was supplied. Furthermore, since the consistency of the milk mixture previously used was unsatisfactory, the proportions were changed to 500 cc. of fresh milk and 250 gm. of whole milk powder. The amounts consumed were not recorded.

The improved performance of the rats after the ration was modified seemed to justify the change. Eight females were mated 90 days before the change was made, and in that time they produced an average of 0.5 litter per animal. In the 90 days after the change was made these same rats gave birth to an average of 1.1 litters per animal. It is well known that the initial productivity of rats is normally higher than was observed in these rats. For example, their fourth generation descendants bore an average of over 1.4 litters per animal in a comparable period. Twenty-three colony rats were mated at about the same time as were the original eight experimental females, and during the first 90 days they bore an average of almost 1.3 litters per animal.

RESULTS

The litter and weaning records of four generations on the experimental ration, and of all animals on the control ration are summarized in figures 1, 2 and 3. Figure 1 shows that at birth the litters of the control animals were significantly larger than those of the experimental group, and that they were still slightly larger at weaning. Also, among the experimental animals there was a tendency in successive generations for the litters to become larger, both at birth and at weaning. It will
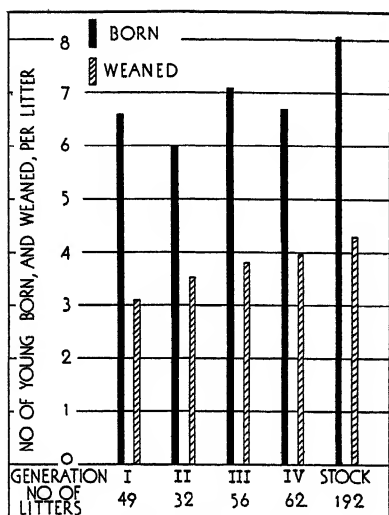
FIG. 1
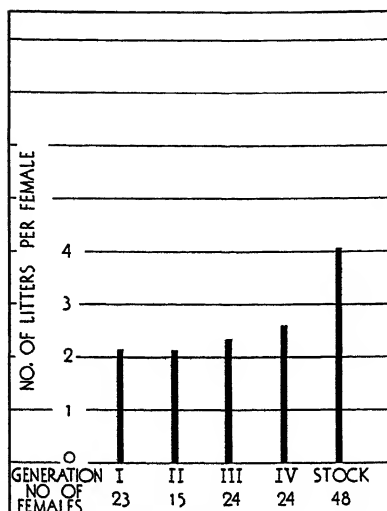
FIG. 2
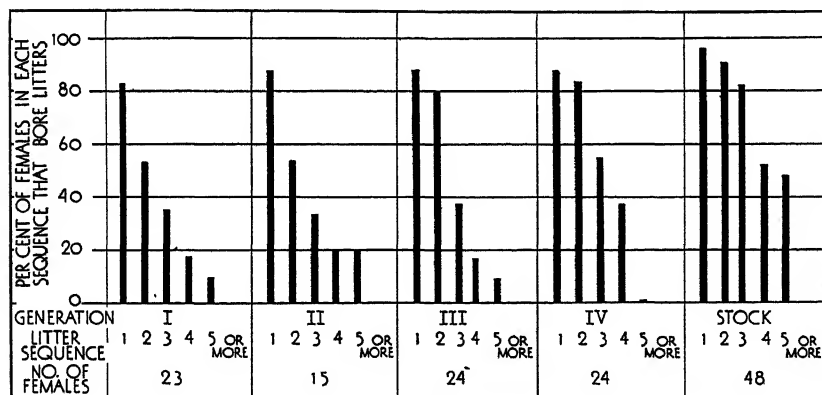
FIG. 3

Fig. 1 Females which received the mineralized milk diet did not bear or wean as many young per litter as did the controls.

Fig. 2 Females which received the mineralized milk diet failed to bear a normal number of litters.

Fig. 3 The ordinate shows the percentage of females in each generation sequence that bore 1, 2, 3, 4 and 5 or more litters. All experimental females produced almost the normal number of first, and the third and fourth generation produced almost the normal number of second litters. In all generations the number of third, fourth and fifth litters was abnormally low.

be recalled that the females of the first generation had been
restricted for the early part of their lives to a low intake of
manganese, and this may account for their inferior perform-
ance. We have no explanation for the observation that the
number weaned by the third and fourth generations continued
to rise. Figure 2 shows that the control animals bore almost
twice as many litters as did those on the milk ration. From
figure 3 it is evident that most of this difference is due to the
smaller number of third, fourth, and fifth litters borne by
the experimental animals. They bore almost the normal
number of first and second litters. Only 20% of the experi-
mental females produced five or more litters, while 48% of
the controls produced that number. It will also be observed
that there was no evident deterioration in succeeding genera-
tions of the experimental animals, the fourth generation
being about as successful as the first.

Since the increase in the amount of minerals, especially of
manganese, was followed by such notable improvement in the
number of litters produced, one may ask if another increase
would have been followed by further improvement. According
to our analysis the solids in the Steenbock ration contain
0.00172% manganese. Peterson and Skinner ('31) report that
the average amount of manganese in milk is 0.028 mg. per
liter. If, then, a rat consumed 20 gm. of the solids and 35 cc.
of milk daily the total daily intake of manganese would still
be less than 0.35 mg. In all probability this estimate is de-
cidedly too high. Inasmuch as the experimental animals re-
ceived almost three times as much manganese as the controls,
one would not expect further enrichment of the milk diet
with respect to this element to be helpful.

Some additional details are shown in table 2. Even with
our system of mating, it would be impossible for a female
to produce a litter much oftener than every 56 days. The
interval among the control animals was 65.5 days, and among
the experimental animals it was approximately 100 days.
The complete data would lead one to expect that under the
same conditions this rate of performance would continue for

some time at least. The ration we used is still deficient though in one or more substances which must be supplied if the females are to bear a normal number of litters.

TABLE 2

Length of experimental period and frequency of litters

GENERATION	LENGTH OF EXPERIMENTAL PERIOD		TOTAL NUMBER LITTERS BORN	AVERAGE INTERVAL BETWEEN LITTERS
	Average	Range		
	days	*days*		*days*
First	218	124–439	49	102
Second	220	70–461	32	103
Third	257	70–433	56	110.4
Fourth	247	133–322	62	95.8
Stock	262	70–428	192	65.5

SUMMARY

1. Four generations of female rats which received milk supplemented with iron, copper, manganese, and iodine, produced and weaned almost as many young per litter as did the controls.

2. The experimental animals produced about half as many litters per female as did the controls.

3. Apparently milk is deficient in some nutrient other than iron, copper, manganese, or iodine, which is essential for normal reproduction.

4. The reproductive performance of the fourth generation was not inferior to that of any of the preceding three.

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BIOLOGICAL ASSAY OF THIAMIN WITH CHICKS

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TWO FIGURES .

Biological assay of thiamin is commonly made with rats. The recent results of Miller ('39), for example, demonstrate that the rat is a highly sensitive test animal in its response to thiamin. The chick is also known to be very sensitive to thiamin deficiency, and a method for bio-assay of thiamin with chicks was described by Arnold and Elvehjem ('38), who reviewed the earlier literature. The present communication describes a method for the assay of thiamin with chicks in which the test diet is evaluated by comparison with a response curve derived by plotting results obtained with graded levels of thiamin. By means of this, the thiamin content of test diets which fail to afford complete protection against polyneuritis may be estimated by measurement of the degree of postponement of polyneuritic symptoms. The use of polyneuritic symptoms, rather than growth rate, for assay purposes has the obvious advantage of specificity.

The problem of supplying all other members of the vitamin B complex in a form free from thiamin has still to be solved. Autoclaved supplements are frequently used for this purpose. Autoclaving has been demonstrated (Jukes, '39) to have a destructive effect upon the filtrate (chick anti-dermatitis) factor (pantothenic acid). However, it was found possible to autoclave yeast to an extent which apparently destroyed the thiamin almost completely, but which permitted the retention of an appreciable proportion of the filtrate factor content.

21

EXPERIMENTAL

A. Preliminary experiments with an autoclaved diet

In the method of Kline and co-workers ('32–'33), the entire cereal and casein portion of the diet is subjected to autoclaving. Experiments were made with a diet similar to that described by Kline and co-workers. It was found that polyneuritis was readily produced on the basal diet, and that polyneuritic symptoms could be prevented by addition of a fullers' earth adsorbate, containing thiamin, to the basal diet. However, when the amount of fullers' earth adsorbate was increased threefold over the level which just protected against polyneuritis, growth was very slow. The chicks developed a crow-like appearance, and their crops became enlarged and hematomatous. Autoclaving is known to reduce the biological value of proteins in cereal grains (Morgan and King, '26). The basal diet of Kline and co-workers was later modified by Arnold and Elvehjem ('38) to contain 2% of untreated vacuum-desiccated whole liver substance. Subsequent investigations in the Wisconsin laboratory placed the thiamin content of dried beef liver at 12 micrograms per gram (Mickelsen et al., '39).

B. Use of an unheated diet supplemented with autoclaved yeast

The diet of Almquist and Stokstad ('35) is known to produce rapid growth in chicks when supplemented with vitamin K to prevent fatal hemorrhages (Almquist, '37). The only source of appreciable amounts of thiamin in this diet is yeast. Autoclaving the yeast should make the diet deficient in thiamin. It was found that autoclaving the yeast used in this investigation at 120°C. for 4 hours destroyed 75% of its filtrate factor content, leaving 6 units per gram. Fifteen per cent of autoclaved yeast was hence included in the basal diet. The basal diet had the following composition (diet 152):

	PARTS
Ground polished rice	68
Washed sardine meal [1]	15
Autoclaved dried brewers' yeast	15
Ground limestone	1
Salt	0.5
Manganous sulfate	0.05
Fish oil blend containing 3000 units of vitamin A and 400 units of vitamin D per gram	0.3
Hexane extract of alfalfa meal, equivalent to one part of alfalfa meal, evaporated on the diet	

Polished rice supplies small amounts of thiamin (Williams and Spies, '38, p. 238). However, it is not possible to obtain satisfactory growth when polished rice is replaced by purified foodstuffs in a diet of this type when fed to chicks. Evidently polished rice supplies a nutritional essential not found to any appreciable extent in yeast (Hogan, Guerrant and Kempster, '25; McFarlane, Graham and Hall, '31). Confirmation of these earlier observations was made in the present investigation.

Synthetic thiamin chloride hydrochloride [2] (abbreviated subsequently to 'thiamin') was dried to constant weight in a vacuum desiccator over sulfuric acid. Solutions were prepared by dissolving 20 mg. of thiamin in 100 cc. of 20% ethanol containing 1 milliequivalent of hydrochloric acid. Aliquots of the solution were mixed in the test diets and allowed to evaporate at room temperature. Ninety-five per cent ethanol was frequently used as a diluent to aid in the mixing process.

Single-Comb White Leghorn chicks were removed from the incubator on the day following the twenty-first day of incubation, placed in electrically heated battery brooders, and fed the experimental diets immediately. From ten to fifteen chicks were used in a group. Growth and food consumption records

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[1] Prepared by washing sardine meal repeatedly with hot water and drying in a large vacuum oven. Kindly supplied by Dr. E. L. R. Stokstad, Western Condensing Company, Petaluma, California. Washed casein may be substituted for washed sardine meal.

[2] Generously furnished by the Merck Institute, Rahway, New Jersey.

were kept. The assay period lasted for 4 weeks. Chicks develop thiamin deficiency so rapidly that no depletion period on the basal diet is needed.

Symptoms of polyneuritis usually first appeared on the ninth or tenth day in chicks on the basal diet, and the birds rarely lived beyond the thirteenth day. Addition of graded levels of thiamin to the diet resulted in the postponement or disappearance of the incidence of symptoms until, at a level of 100 micrograms of added thiamin per 100 gm. of diet, protection was usually complete during the 4-week assay period. Individual variation was so marked that it was considered advisable to use at least ten chicks in each test group. Birds were closely watched for symptoms of polyneuritis, characterized by opisthotonos and inability to stand. Death usually followed within 1 or 2 days. Loss of weight always preceded the appearance of polyneuritic symptoms. Some birds died, often during the night, without polyneuritic symptoms being noted. Such birds were eliminated from calculation. The birds dying with polyneuritic symptoms, together with birds surviving the assay period, were used to calculate the 'polyneuritic mortality index' of the test group. The index value of a bird was calculated by subtracting the number of days it survived from the number of days (28) in the assay period. These values were averaged to give the index value of the test group. Table 1 illustrates the method of calculation. The index value of the group is obtained by adding the figures in the last row and dividing by the number of chicks (12) to give a value of 3.2.

A diet which gave complete protection thus gave an index value of zero, and the index value of the basal diet was usually between 16 and 17. In the fall months this value may rise to about 19, with corresponding increases in the index values of supplemented diets. The indication is that there was less thiamin deposited in the egg during the fall months, since the diet of the parent hens was unchanged. Table 2 summarizes a number of the index values and figure 1 illustrates the response curve obtained by plotting index values against the

TABLE 1

Illustration of the method of calculation. Group no. 152-28.
Supplement = 70 micrograms thiamin per 100 gm. diet. Assay period = 28 days

Bird no.	1	2	3	4	5	6	7	8	9	10	11	12	13
Age at which polyneuritic symptoms first observed (days)	21	16	a	a	a	a	23	27	15	a	20	a	a
Age at death (days)	22	17	b	b	b	b	26	b	15	b	22	b	8
Number of days of survival subtracted from number of days in assay period [c]	6	11	0	0	0	0	2	0	13	0	6	0	d

a   Polyneuritic symptoms not observed.
b   Survived the assay period.
c   This value is the 'polyneuritic mortality index.' Birds surviving the assay period receive a value of zero, indicating complete protection.
d   Eliminated from calculation, since death was not observed to be preceded by polyneuritic symptoms.

The index value of the group is obtained by adding the figures in the last row and dividing by the number of chicks (12) to give a value of 3.2.

TABLE 2

Polyneuritic mortality indices obtained with varying levels of thiamin

AMOUNT OF THIAMIN ADDED TO 100 GM. OF BASAL DIET 152	POLYNEURITIC MORTALITY INDICES							TOTAL NUMBER OF CHICKS INVOLVED
micrograms	Expt. 1	2	3	4	5	6	Average	
0	16.0	16.8	17.0	16.7	17.3	18.0	17.0	77
30				12.3	15.9		14.1	30
40				10.7	14.2		12.4	30
50	7.9		10.5	6.5	12.1	9.1	9.2	64
60		3.0			9.2		6.1	25
70	1.8		3.2			3.4	2.8	34
80		2.7					2.7	10
90			0		1.5		0.8	27
100	0	0					0	22
120		0	0			0	0	37
150	0						0	12

level of thiamin added to the diet. It will be seen that the method reaches its maximal sensitivity in the region between 30 and 70 micrograms. In conducting an assay, therefore, an amount of the test material expected to furnish an increase of about 50 micrograms of thiamin is added to 100 gm. of the basal diet. In this region the average food consumption per bird per day is about 5 gm. and a difference of 10 micrograms of thiamin per 100 gm. of diet is sufficient to produce a definite change in the index. This difference corresponds to an intake of less than 1 microgram of thiamin per bird per day.
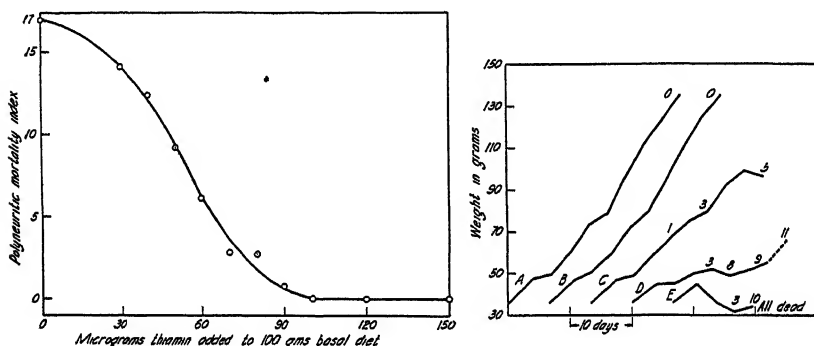
Fig. 1   Response curve obtained by plotting average polyneuritic mortality indices, from table 1, against levels of thiamin added to diet 152.

Fig. 2   Growth curves of chicks in experiment 1. Twelve chicks were used in each group. The thiamin supplement added to 100 gm. of basal diet was for group A, 150 micrograms; B, 100 micrograms; C, 70 micrograms; D, 50 micrograms; E, none (basal diet). The numbers above the curves represent the cumulative total of observed cases of polyneuritis. The last part of the growth curve of group D is represented by a broken line because only two birds survived after the eleventh day. All the chicks in group E were dead by the fourteenth day.

C. Growth and food consumption

Figure 2 illustrates the growth in experiment 1. The average daily food consumption per bird, obtained by dividing the total food consumption by the sum of the number of days of survival of each chick, was for group A, 11.8 gm.; group B, 11.7 gm.; group C, 6.9 gm.; group D, 3.8 gm.; group E, 1.0 gm. Part of the diet was placed on the floor of the cage on sheets of paper for the first 4 days to encourage the chicks to start

eating, and hence the first 4 days were eliminated from calculation of food consumption due to wastage of diet. The food consumption data well illustrate the marked effect of thiamin deficiency upon appetite. Figure 2 shows that the growth of chicks was not improved by increasing the level of thiamin in the diet beyond the level (100 micrograms) which just protected against polyneuritis. This conclusion was supported by the results of the other experiments.

TABLE 3

Assay of polished rice for thiamin content

AMOUNT OF THIAMIN ADDED TO 100 GM. OF GLUCOSE DIET	POLYNEURITIC MORTALITY INDEX: EXPERIMENT		THIAMIN ADDITION TO RICE DIET CORRESPONDING TO THE PRECEDING INDEX, OBTAINED FROM FIGURE 1	DIFFERENCE BETWEEN GLUCOSE AND RICE DIETS, IN TERMS OF THIAMIN PER 100 GM. OF DIET
micrograms	1	2	micrograms	micrograms
0		21.6	a	
30	17.1		0	30
40	15.2		23	17
50	14.0		30	20
70		11.2	43	27
90		7.3	57	33
120	2.3	1.9	79 [b]	41
150		0	Complete protection	..

a Beyond lower limit of figure 1.
b From the average value of the two indices.

D. Estimation of thiamin content of polished rice

Glucose [3] was substituted for polished rice in the basal diet, and various levels of thiamin were added. The index values obtained were compared with the response curve (fig. 1) with the results shown in table 3.

The average value of the difference between the glucose and rice diets is 28 micrograms per 100 gm. of diet. Assuming that the glucose was free from thiamin, it may be deduced that 68 gm. of polished rice contained 28 micrograms of thiamin, corresponding to 0.4 micrograms of thiamin per gram.

[3] 'Cerelose.'

Growth was slow on the glucose diet even when completely supplemented with thiamin. The chicks in the group receiving an addition of 150 micrograms per 100 gm. of diet averaged only 63 gm. in weight at 28 days of age. Groups of chicks which received the rice diet completely supplemented with thiamin weighed from 110 to 134 gm. at 28 days. This result may be compared with the observations of Hogan, Guerrant and Kempster ('25) who found that chicks receiving a ration composed of starch, casein, salt mixture, yeast and cod liver oil grew slowly, but that growth was markedly accelerated when the corn starch was replaced by polished rice. Similar results were noted by McFarlane, Graham and Hall ('31) and by Stokstad and Manning ('39). For this reason, even though the glucose diet was more deficient in thiamin, it was thought desirable to use the rice diet for regular assay purposes. Substitutions for assay purposes may be made in the rice diet with the assumption that polished rice supplies 0.4 micrograms of thiamin per gram. Since an addition of 100 micrograms was just sufficient to protect the birds on diet 152, the minimal thiamin requirement of the chick was at least 128 micrograms per 100 gm. of diet for the first 4 weeks under the experimental conditions involved, basal diet 152 supplying at least 28 micrograms of thiamin per 100 gm. due to its content of polished rice. The amount of thiamin supplied by other constituents of the diet is presumably quite small, and the minimal thiamin requirement of the chick may probably be estimated as between 130 and 150 micrograms per 100 gm. of diet.

DISCUSSION

The response curve illustrated in figure 1 appeared to be fairly constant for the chicks used in the present investigation. Others who use the method may wish to construct response curves under their own conditions. For this purpose, it is suggested that in making an assay the following supplements should be fed to test groups: group 1, none (basal diet); group 2, 30 micrograms; group 3, 50 micrograms; group 4,

70 micrograms; group 5, 100 micrograms; group 6, 150 micrograms of thiamin per 100 gm. of diet; group 7, enough test substance to furnish about 50 micrograms; group 8, enough to furnish about 100 micrograms per 100 gm. of diet. Groups 2 and 6 are less important than the other groups. Substitutions may be made for a part of the polished rice, assuming a content of 0.4 micrograms of thiamin per gram for this material in calculating the results. If the material to be tested is high in protein, part of the fish meal in the basal diet may be replaced. In the case of materials unusually low in thiamin, it may not be possible, of course, to supplement the diet with levels of the material furnishing as much as 50 micrograms of thiamin per 100 gm. of diet. In such a case, it is necessary and feasible to use the upper portion of the response curve in calculating the results.

The gregarious eating habits of the chick are advantageous in biological assays in that a group of chicks will usually feed in comparative unison. Food spillage is small with suitably constructed troughs. It is particularly desirable to examine the chicks for symptoms of polyneuritis in the late evening during the final 3 weeks, since cases frequently develop during the evening and terminate fatally before the next morning.

The method described in the present communication has been used in the assay of muscular tissue from pigs fed diets containing various levels of thiamin (Hughes and Heitman)[4], in a study of thiamin in the diets of ruminants (McElroy and Goss, '39) and in the assay of commercial dog food for thiamin (Caldwell).[5]

SUMMARY

1. A method is described for the assay of thiamin with chicks, using a basal diet consisting principally of polished rice, fish meal and autoclaved yeast.

2. A response curve was constructed in which the level of thiamin added to the diet is compared with the 'polyneuritic mortality index,' the latter value being expressive of the de-

[4] To be published.
[5] Personal communication.

gree of protection afforded by a suboptimal level of thiamin fed during the assay period of 28 days.

3. The thiamin requirement of the chick was estimated as being between 130 and 150 micrograms of thiamin chloride hydrochloride per 100 gm. of diet for prevention of polyneuritis under the conditions studied.

4. The thiamin content of the polished rice used in the basal diet was found to be about 0.4 micrograms per gram. A growth essential for the chick, absent from autoclaved yeast, was found present in polished rice.

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PREVENTION OF HYPERPLASIA IN THE FORE-STOMACH EPITHELIUM OF RATS FED WHITE FLOUR

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Hyperplasia of the squamous epithelium in the forestomach of rats has been the subject of numerous studies since Pappenheimer and Larimore ('24) found the lesions in rats fed a diet of white flour. The similarity of the papillomatous changes to the non-malignant type produced by Fibiger ('19) with Spiroptera was recognized and they suggested that an irritating substance was ingested. It was found that the rats were plucking and eating their hair and that hairs could be found embedded in the abnormal epithelium. However, ingestion of hair by normal animals did not produce the changes, which led Pappenheimer and Larimore to conclude that an essential dietary factor was missing in addition to the presence of an irritating substance. Recent dietary studies (Passey, Leese and Knox, '35) suggest that the lesions obtained in rats and mice by Fibiger depended to a large extent upon his diet of white bread. There is now general agreement that the lesions are induced by an incomplete diet but there is no agreement on the nature of the dietary fault. A number of authors suggest that vitamin A deficiency produces the lesions and quote the work of Fujimaki ('26). Yet, Fujimaki, who first reported the effect of vitamin A deficiency, found later ('31) that it was unimportant. Howes and Vivier ('36) demonstrated that yeast will prevent the lesions but, in contrast to the studies of Hoelzel and DaCosta ('37) who ascribe the lesions to a deficiency of biologically good protein,

31

found that a diet fortified with 20% casein will not provide protection. Findlay ('28) produced the lesions with vitamin B_2 deficiency but not with B_1 deficiency.

In previous studies (Sharpless, '37) with a purified low-protein diet, extreme hyperplasia occurred which was prevented by feeding cystine. However, the need for further study was evident when cystine failed to prevent the lesions in rats fed the white flour diet.

EXPERIMENTAL PART

The general plan of the experiment was to study, in rats fed a white flour diet, the effect of cystine and the vitamins known to be in yeast upon the development of gastric lesions. Two preliminary experiments showed that a deficiency in the protein of white flour was not the causative factor. First, the white bread flour diet fortified with 10% of vitamin free casein did not prevent the lesions, which confirmed the same finding of Howes and Vivier ('36). Second, fortification with 10% of gelatin together with the necessary amino acids to make it a complete protein did not prevent the lesions.

Each experiment in the present series extended over a 6-week period. In all cases young rats, both male and female, weighing (with few exceptions) between 60 and 80 gm., were kept in wire cages and were provided with food and water ad lib. Except for the addition of 5% butterfat and viosterol, the basal diet was the same as that used by Pappenheimer and Larimore ('24). Litter mate controls were fed the basal diet in each experiment.

The following concentrates, in all possible combinations, were added to the basal diet: 'Labco' desiccated rice polish concentrate, lactoflavine, nicotinic acid, and cystine. The composition of the diets is given in table 1.

RESULTS

None of the diets which contained only one concentrate prevented the lesions, whereas the diet which included all of them gave almost complete protection. The results are

recorded in table 2. The lesions have been well described by Pappenheimer and Larimore ('24) and also by Howes and Vivier ('36). All animals with one or more hyperplastic areas visible to the naked eye were recorded as having lesions. No attempt was made to record in the table differences in severity of the lesions.

Of twenty-five rats fed diet 229, which contained all four supplements, just one had a papillomatous area and it was

TABLE 1

Composition of diets [1]

DIET NO.	WHITE BREAD FLOUR	RICE POLISH CONCENTRATE	LACTO-FLAVINE	CYSTINE	NICOTINIC ACID	VITAMIN B1
	%	%	μg.%	%	%	%
151	90.0	...	...	...	...	
152	90.0	...	100	...	...	
154	89.0	1.0	...	...	...	
175	88.0	2.0	200	...	...	
177	87.5	2.0	200	0.5	...	
178	87.5	2.0	...	0.5	...	
190	89.5	...	100	0.5	...	
191	89.5	...	...	0.5	...	
199	89.0	...	...	...	1.0	
229	88.0	1.0	100	0.5	0.5	....
236	89.0	...	...	0.5	0.5	....
237	89.0	...	100	0.5	0.5	....
238	88.0	1.0	...	0.5	0.5	....
239	88.5	1.0	...	...	0.5	....
240	89.5	...	100	...	0.5	....
251	88.5	1.0	100	...	0.5	....
269	89.0	...	100	0.5	0.5	0.002

[1] Each diet contained in addition, 5% butterfat, 2.9% calcium lactate, 2% sodium chloride, 0.1% ferric citrate and 15 drops of viosterol per kilo.

very small. This diet was the only one which provided such complete protection although diet 251 which contained all of the supplements but cystine produced one or two small papillomatous areas in each of five rats from a group of nineteen. Diet 154, where the supplement was rice polish concentrate, suggests some protection, but the controls did not show a high incidence of lesions and a diet containing 2% of the concentrate provided no greater protection than

that obtained from 1%. The purpose of the experiment was to obtain complete protection; therefore, it seemed useless to study larger groups of animals on diets which indicated only slight protection. Furthermore, partial protection is very difficult to determine because there is variation in incidence of lesions between litters and between lots of flour.

In order to determine whether vitamin B_1 was the active factor in rice polish concentrate, another diet (269) which

TABLE 2
Average body weight and incidence of gastric lesions

DIET	AVERAGE WEIGHT				NUMBER OF ANIMALS			
	Experimental animals		Controls		Experimental animals		Controls	
	Start	Final	Start	Final	Total	With lesions	Total	With lesions
	gm.	*gm.*	*gm.*	*gm.*				
151	..	..	69	65	..	..	155	132
152	57	63	57	72	6	4	6	3
154	70	77	71	84	13	5	9	5
175	72	93	69	67	5	4	4	4
177	63	73	63	56	4	2	6	6
178	65	71	63	56	4	4	6	6
190	58	59	55	51	6	6	3	2
191	68	57	62	43	5	3	3	3
199	74	74	76	61	4	2	3	2
229	67	74	67	57	25	1	22	22
236	65	55	68	65	6	3	3	3
237	85	80	85	79	6	6	3	3
238	70	68	66	53	6	3	3	3
239	68	68	74	62	5	2	3	2
240	76	70	76	66	5	4	3	2
251	70	77	70	60	19	5	15	15
269	87	82	87	82	10	9	10	5

contained 2 mg. per cent of thiamine in place of the 1% rice polish concentrate in diet 229 was studied. Nine of the ten rats fed this diet had lesions. Therefore, the essential supplements to prevent the lesions are lactoflavine, cystine, nicotinic acid, and at least one factor other than vitamin B_1, contained in rice polishings.

The effect of the supplements on food intake and the resultant effect on growth bears no relation to production of

the lesions. Rats fed diet 229 grew better than their controls but no better than those fed diet 154, 175 or 178, none of which prevented the lesions.

These experiments along with previous studies (Sharpless, '37) show that the lesions in the forestomach are prevented by a non-specific factor. In a low protein diet cystine may be the preventive factor. In a diet of white flour, cystine plays a minor role and all of at least three members of the vitamin B complex are necessary to prevent the lesions. This non-specific property of the protective substance clarifies some of the confusion that has arisen from studies in which a specific factor was thought to be the protective substance.

The prevention of lesions in the forestomach by a non-specific factor raises two questions which should be discussed in the light of available evidence. First, why do the changes occur in the squamous epithelium of the forestomach and not in other areas of squamous epithelium? Second, is there any evidence that the protective factors have in general any metabolic interrelationship or in particular, any relation to the metabolism of epithelial tissue?

Although the epithelium of the bladder, vagina and pelvis of the kidney is also of the squamous type, there is no sign of lesions in any of these tissues. If the metabolism of squamous epithelium in different parts of the body is the same, the assumption must be made that local conditions account in part for the lesions in the forestomach. Hoelzel and DaCosta have advanced the theory that the primary cause of the lesions is the irritating action of gastric juice. Other studies of our own in which pepsin was fed with and without hydrochloric acid in the drinking water, showed that increase in acidity and pepsin could not completely explain the extreme hyperplasia that occurred with some of the diets. Therefore, regardless of conditions that may be produced in the stomach by the diets studied, it appears that the epithelium has been changed so that less stimulus is required to produce

proliferation. In other words, the threshold of stimulation to proliferation of the epithelial tissue has been lowered.

Evidence that the protective factors have a metabolic inter-relationship and that they affect the sensitivity of the cell to proliferative stimuli is fragmentary but certain reports indicate that such relationships may exist. Hueper ('34) has shown that there are many instances in which epithelial tissue is more sensitive to proliferative stimuli when its sulphydryl content is reduced. A decrease in glutathione (and there-fore a decrease in sulphydryl) content of various tissues of the rat results from feeding a diet deficient in cystine (Marenzi and Braier, '34) or a diet deficient in vitamin B_2 (Itter, Orent and McCollum, '35). Vitamin B_2 is now known to consist of at least three fractions, nicotinic acid, lactoflavine, and vitamin B_6. These are three of the protective factors; although vitamin B_6 was not isolated, it is the other vitamin that is known to be present in abundance in rice polishings. The fact that nicotinic acid cures pellagra with its associated low cystine content of finger nails suggests a relationship between nicotinic acid and sulfur metabolism of epithelial tissue. Recent studies of biological oxidation reduction systems show that the system containing nicotinic acid and the one containing lactoflavine may be concerned with two different phases of metabolism of the same material. Vitamin B_6, since it is the rat antidermatitis factor, is known to be concerned with the sensitivity of epithe-lial cells to proliferative stimuli. This evidence suggests that the non-specific factors prevent the lesions by lowering the sensitivity of the cell to proliferative stimuli and that some of the factors may do this by increasing the sulphydryl content of the cell.

SUMMARY

Hyperplasia of the squamous epithelium in the forestomach of rats fed a diet of white flour is prevented by adding lacto-flavine, nicotinic acid, cystine, and rice polish concentrate to the diet. With a diet of white flour, protein or starvation are not important factors in production of the lesions. The

relation of local irritation to formation of the lesions and the relation of the non-specific protective factors to sensitivity of epithelial cells to proliferative stimuli is discussed.

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THE STATE OF VITAMIN A IN THE LIVER OF THE RAT AFTER FEEDING VARIOUS FORMS OF THE VITAMIN

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TWO FIGURES

INTRODUCTION

In the fish liver, vitamin A occurs as an ester. This was demonstrated for cod in 1928 by Bacharach and Smith who employed aqueous methyl alcohol and petroleum ether to separate the two forms. Employing this same method, Reti, in 1935, showed that the vitamin is present in the ester form in the livers of fish, chickens, and various mammals. In 1936, Hickman, employing distillation and the technique of the elimination curve, showed that in both cod and halibut liver oils, the vitamin A has been largely esterified. Since then, analysis by distillation has been extended to a sufficient number of different species of fish to warrant the generalized statement that vitamin A occurs in all fish-liver oils in the ester form, sometimes accompanied by a trace of the free vitamin. The latter is believed to arise after the death of the fish and to be generated by hydrolysis due to rancidity, the alkali used in refining, or other causes.

It seemed of interest to extend the investigation to mammals in order to obtain some information as to the state of the vitamin stored in the liver and the effect of feeding the different kinds of vitamins. Presumably, the mammal is dependent upon the diet for its vitamin A. Vitamin A can occur in the

diet in three well-defined forms, namely, provitamins, including beta carotene and the other carotenoids, the various esters of vitamin A, and lastly free vitamin A itself. There is thus a clear-cut problem. Does each kind of vitamin in the diet produce a separate selection of vitamin bodies in the liver or do all varieties become stored as the same selection of esters?

It was felt that the analytical technique of molecular distillation was excellently suited for providing an answer to these questions. Since each provitamin or vitamin compound distills at a characteristic temperature identified by the temperature of the elimination maximum, it is possible, by inspecting a distillation map of vitamin A recovered from the liver, to tell at a glance approximately what selection of esters is present and how these differ from the materials administered. For the exploratory work described in this paper, rats were used and the vitamin preparations were administered in massive doses over a short period. It is realized that this procedure is not ideal, and it is planned to extend the study with doses nearer the physiological level. Shortly after feeding, the rats were killed, the livers were removed, and the oil extracted and distilled. Vitamin A was fed in six different forms, as enumerated in the experimental part.

EXPERIMENTAL PART

Thirteen litters of young white rats from the Sprague-Dawley strain were reared on the Sherman B diet (Sherman and Campbell, '24), the vitamin A in this diet being obtained from whole milk. This ration is sufficient for the early growth, but rats reared on this diet do not store vitamin A in their livers in excessive amounts (McCoord and Luce-Clausen, '34). For our purposes, therefore, we can regard this diet as borderline from the standpoint of liver storage. It was chosen for this reason.

At the age of 53 days, the rats were sampled from each litter and distributed among seven groups to insure approximately equal weight distribution (Luce-Clausen, '29). The vitamin oils were administered by dropper until 20,000 units

of vitamin A had been fed in the various forms during 48 hours. The volume fed varied in each sample group as follows:

U.S.P. reference oil [1]	329 drops
Vitamin A caproate	23 drops
Natural ester concentrate [2]	23 drops
Vitamin A stearate	22 drops
Vitamin A alcohol	20 drops
Beta carotene	212 drops

The animals were then killed, the livers removed, and those from each group were pooled and weighed. They were then frozen and kept at — 55°C. until used. On examination, each group was thawed and ground up with anhydrous Na_2SO_4 and then extracted with ether. Four hundred cubic centimeters of ether were used for the first extraction of each batch, 200 cc. being used for successive extractions. Eight extractions were sufficient to recover substantially all of the vitamin A. The ether solutions were filtered, and the ether removed. With the earlier groups, constant yield oil and residue [3] were added before all of the ether was removed, to minimize destruction of the vitamin. This precaution was found to be unnecessary and so the later samples were taken to dryness, weighed and analyzed before addition of the oil. It was found necessary at some time during the procedure to treat the fatty extract with 50 cc. of n/10 alkali to remove certain nitrogenous constituents, which, if left in the oil, caused spluttering on the still column and interfered with the distillation. The easiest method of treatment was to add the alkali directly to the extract before addition of diluent oil. This material was then shaken out with ether, the ether solution washed with distilled water and dried with anhydrous Na_2SO_4 and the ether removed. If the material was alkali-treated after addition of the diluent oil, difficulties with emulsion formation were encountered which made the separation extremely tedious, ac-

[1] In this group the actual dose was 5.8% short due to the refusal of the rats to take the full dose.

[2] Distilled without modification from a rich fish-liver oil.

[3] A high-boiling fraction, free from vitamin A, obtained from the complete distillation of a fish oil.

companied by a certain amount of mechanical loss of oil solution.

After dilution of the extract with constant yield oil and residue, the oils were distilled, using the technique employed in this laboratory (Hickman, '37; Embree, '37). Total vitamin content of each fraction was plotted against the temperature at which the fraction distilled. Curves are given in figure 1. Vitamin A stearate and caproate and distilled ester concentrate furnished similar curves which overlapped in many cases.

Figure 1
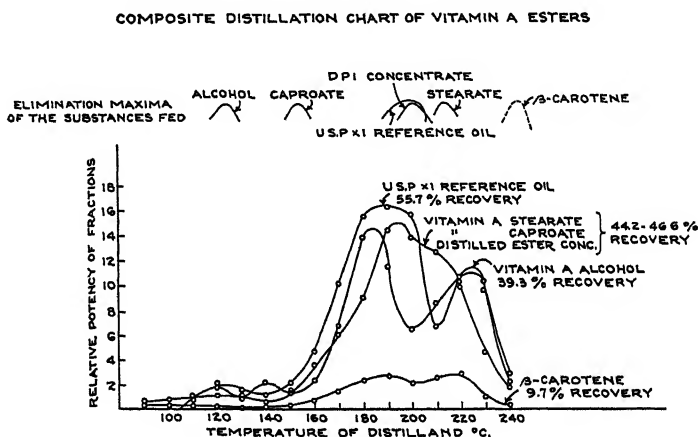
To present a less confusing picture, they have been plotted as one curve.

The vitamin was assayed by spectrophotometer and by the Evelyn colorimeter. Unfortunately many fractions were too low to display a good absorption spectrum in the ultra-violet, and so the $SbCl_3$ blue color was used in these cases. Where potencies were sufficiently high for spectrophotometric analysis in the ultra-violet, good agreement was obtained with the two methods. The results are summarized in table 1.

DISCUSSION

The positions of the elimination maxima of the five forms of the vitamin originally fed are indicated at the top of figure

1 for comparison purposes. Since beta carotene was measured as vitamin A, the elimination maximum of the carotene as such is of secondary importance. Only traces of unchanged carotene were recovered from the livers. A small but definite quantity of free vitamin A alcohol is present in all the groups and shows as low maxima at about 120°C. on the curves. As there was not sufficient vitamin present in the control group

TABLE 1

The recovery of vitamin A in the liver of the rat after feeding various forms of the vitamin

SUBSTANCE FED	WEIGHT OF RATS[1]		WEIGHT OF LIVERS		WEIGHT OF EX-TRACT	TOTAL VITAMIN A FED	TOTAL VITAMIN A[3] RECOVERED	RECOV-ERY
	Total	Av. wt. per rat	Total	Av. wt. per rat				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*I.U.*	*I.U.*	*%*
U.S.P. XI reference oil	1962	131	94	6.7 [2]	3.8	273,000	152,000	55.7
Vitamin A caproate	1971	131	87	5.8	..	292,000	136,000	46.6
Distilled ester concentrate	2001	133	94	6.3	3.3	288,000	127,400	44.2
Vitamin A stearate	2030	135	88	5.9	..	300,000	132,800	44.3
Vitamin A alcohol	1996	133	84	5.6	3.45	300,000	118,000	39.3
Beta carotene	2045	136	92	6.1	..	287,000	27,700	9.7
Controls	1323	132	58	5.8	1.8		4,500	

[1] There were fifteen rats in each group except for the controls which had ten rats.

[2] One rat in this group died so the figures for all but weight of rats are based on fourteen rather than fifteen rats.

[3] Due to the potencies involved, the limits of error on the determination are ±15%.

for a distillation, the alcohol: ester ratio was determined by partitioning the lipid between aqueous methyl alcohol (83%) and petroleum ether (Bacharach and Smith, '28; Reti, '35). Approximately 90% of the vitamin was found to be esterified, with 10% in the free form. The small amount of free vitamin always recovered from these rat livers may or may not be significant. Its presence may prove to be as accidental as it

has in the case of the fish-liver oils, but this is considered to be unlikely since it occurs in larger proportions. It seems probable that the A-alcohol serves some physiological purpose not met by the esters or the provitamins. This phase is a subject for further investigation.

The percentage recovery presents a suggestive picture. Since 90–95% of the absorbed vitamin appears in the liver, the vitamin A in this organ may be taken as a measure of vitamin A absorption (McCoord and Luce-Clausen, '34; Moore, '31; and Baumann et al., '34). More accurate data are necessary to prove the exact order of percentage utilization. However, a trend is in evidence with the greatest recovery exhibited from U.S.P. XI reference oil, followed by the separated esters, then the A-alcohol, with beta carotene running a very poor last. The poor utilization of the carotene was visible, large amounts of unchanged provitamin showing in the faeces. Upon autopsy, the entire gastro-intestinal tract appeared bright orange from the pigment. Admittedly large amounts of fat were fed with the carotene, but even larger amounts were fed in the form of U.S.P. XI reference oil which appeared to be the most readily utilized of all. While at small dosage levels, vitamin A and carotene are apparently completely absorbed and utilized, as the dosage is increased, the absorption of carotene and vitamin A fall off, that of carotene falling much lower than that for vitamin A. Davies and Moore ('34), feeding very high levels of vitamin A and carotene, found 15% of the vitamin A absorbed and but 1% of the carotene. Further work on the recovery of these substances in the liver after ingestion at much lower levels will doubtless show whether or not the substances normally fall in the order listed.

A further point of interest is that there is a dip in the ester portion of the elimination curve of the vitamin A recovered from the rat livers, whereas there is no dip in similar curves from the distillation of fish-liver oils. The dip is greatest in the case of cod liver oil, the A-alcohol, and beta carotene; and least in the case of the other esters which give inflexions instead of minima. These curves should be compared with that

for lingcod-liver oil, which is shown in figure 2. There is no minimum here but a trace of inflexion at 200°C., and the curve follows through a maximum at about 220° and falls off. The curve is too broad to denote a simple substance, but evidently represents a whole series of esters, as indeed one would expect to find upon examination of the fatty acids of the glycerides themselves. In contrast to this, the curves from the rat liver oil suggest that some of the fatty acids of intermediate molecular weight are missing, or present only in small quantity. In other words, the liver selects certain specific acids with which to esterify the vitamin to the partial or complete
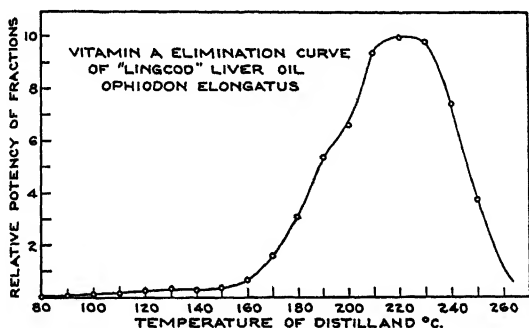
Figure 2

exclusion of others. This is purely a tentative suggestion and may be modified by later findings.

SUMMARY

1. Vitamin A, fed to rats in the following forms—

    U.S.P. reference oil
    Vitamin A caproate
    Distilled ester concentrate
    Vitamin A stearate
    Vitamin A alcohol
    Beta carotene

was recovered in the livers as the esters, the percentage recovery being in the order of the substances listed. Fifty-five and six-tenths per cent of the vitamin fed as U.S.P. reference

oil was recovered, as against 39.3% recovery from that fed as alcohol and 9.7% recovery from beta carotene.

2. A small amount of the vitamin alcohol was always present, the significance of which is at present not known.

3. The type of ester in each case was similar, which suggests a selective utilization of fatty acids by the rat for the purpose of esterifying the vitamin.

ACKNOWLEDGMENT

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CHOLINE IN THE DIET OF CHICKENS

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The attention of nutritionists for many years has been directed to the identification of nutrients necessary for growth and reproduction of chickens. Much progress has been made on both the qualitative and quantitative demands but the problem is still one of major interest. Very early in these investigations it was found that simplified diets adequate for rats were inadequate for the satisfactory nutrition of the chick.

In the study of simplified diets Hogan, Boucher and Kempster ('35) described rations which apparently contained all nutrients required by the chick. One of the most promising of these consisted of casein, cornstarch, cellophane, tikitiki, lard, salt mixture, wheat germ oil, acid hydrolized yeast, liver extract, $CaCO_3$; and in addition each chick received daily 0.2 mg. crystalline carotene, and 240 International Units of irradiated ergosterol. Four successive generations were reared on simplified rations. The authors state that the chicks grew rapidly. The males were normal in appearance throughout the period of observation, but their fertility was low; the females were normal until they obtained maturity, but after periods of intensive laying the mortality was high. Their egg records, however, compared favorably with those obtained under normal conditions. From this work it was concluded that if, as indicated by the work of others, the low fertility of the males and the high mortality of the females could be explained on the basis of rearing under laboratory conditions, then the simplified diets were complete in all respects.

47

It will be noted that while the above diet did not contain lipoids as such, they undoubtedly were present in the extracts of liver and yeast. Moreover, these extracts were found by Hogan, Boucher and Kempster ('35) to be necessary adjuncts to the diet. In the preparation of purified diets it is assumed, however, that animals are able to synthesize lecithin and other lipoids containing phosphorus in large amounts. This assumption is based primarily on the work of McCollum, Halpin and Drescher ('12) and on that of Fingerling ('12).

The recent investigations of Best and his collaborators have stimulated interest in lecithin and its compounds. Best, Hershey and Huntsman ('32) showed that rats fed a daily ration containing 2.5 gm. of fairly saturated fats may produce in the livers large amounts of fatty acids, but if lecithin were added to the diet the liver fat was not in excess of that seen in animals fed a normal diet. Best and Huntsman ('32) then fed separate components of lecithin, but of these, only choline consistently inhibited the deposit of fat in the livers. Later, Best, Huntsman, McHenry and Ridout ('35) found that when rats were fed a ration which provided adequate amounts of protein, fats, carbohydrates and vitamins A, B_1, B_2, B_4 and D, large amounts of fat rapidly accumulated in the livers if the diet did not contain appreciable amounts of choline, or substances having similar effect on liver fat. In this paper evidence was presented which indicated that choline was an accessory food factor.

According to Mathews ('23) egg yolks contain 11% of lecithin, so that 3 gm. of lecithin are necessary for an average yolk. Because of the relatively large lecithin requirement for egg production, the question of lecithin synthesis in the hen is one of importance.

EXPERIMENTAL

The object of this investigation was to determine the effect of lecithin or choline on the nutrition and egg production of chickens when added to the McCollum, Halpin and Drescher milk-rice diet supplemented with the necessary vitamins.

Experimental chicks. Chicks from a high producing strain of Rhode Island Reds incubated in the laboratory of the Department of Home Economics were used in this investigation. They were fed a commercial starting mash until they were 1 week old and from then on the starting mash was gradually replaced by the experimental ration. This usually required about 2 weeks. In the experimental work with pullets the birds were fed the growing mash until they were 16 weeks old and the laying mash until placed on the milk-rice ration at 4 to 6 months of age.

Quarters. When the birds were placed on experimental rations they were weighed and put into individual wire cages which were enclosed in a larger tightly-screened, insect-proof cage. This cage had a metal roof but the sides were covered with fine meshed copper wire, allowing the birds to be in the sun the greater part of the day.

Rations. The chief components of the basal ration were essentially the same as those used by McCollum, Halpin and Drescher ('12) in their study of lecithin synthesis in the hen—skim milk powder (Merrill-Soule) and polished rice. To these items were added the necessary vitamins, granite grit, oyster shell and cellulose. Following the procedure of McCollum and his associates the rice was ground to a powder and then extracted for 30 minutes with boiling alcohol. Two extractions were made, after which the rice was transferred to a press and nearly freed from alcohol. Finally it was spread in a thin layer until the remaining alcohol evaporated. This diet was considered practically lecithin-free. McCollum found that the ether extract of the milk powder contained about 0.6% of ether-soluble matter and was practically free from phosphorus, while the extracted rice was also relatively fat- and lecithin-free. After a number of experiments to determine the vitamin requirement for pullets, the following diet was found to give the best results: skim milk powder (Merrill-Soule), 30%; rice powder 70%. The vitamin supplements given daily were: 2428 U.S.P. units vitamin A, 485 U.S.P. units vitamin

D, 416 Sherman units vitamin B_1, 326 Sherman units of B_2 complex.

In feeding chicks, one-fourth of the daily vitamin requirement for pullets was used for the first month and after that the number of vitamin units was gradually increased until the chicks were 3 months old. At that time they were receiving the pullet ration. The lecithin and choline supplements will be given with the experimental data.

Chemical methods. The chickens were killed by cutting the carotid artery and the livers removed immediately. The procedure and methods of Best, Channon and Ridout ('34) were followed in the preparation of the samples, and the fatty acids prepared as described by Channon and El Saby ('32).

TABLE 1

Egg production and condition of 5 to 6 months old Rhode Island Red pullets fed from 195 to 260 days on a simplified diet plus lecithin

NUMBER OF PULLETS	DIET	NUMBER OF DAYS ON DIET WHEN LAYING BEGAN	NUMBER OF DAYS ON DIET	AVERAGE NUMBER EGGS LAID PER BIRD	REMARKS
12	Basal	30–70	195	16	3 hens died; others stopped laying.
20	Basal + 1 gm. lecithin	20–60	260	50	Hens in fair condition; continued laying.
10	Laying mash	20–60	275	77	In excellent condition; still laying.

RESULTS

The results of feeding chicks and pullets lecithin or choline as an adjunct to a basal diet composed of dried skim milk, rice and vitamins, and the effect of choline on the fat content of the livers, are summarized in tables 1, 2, and 3.

Rhode Island Red pullets 5 to 6 months old fed the basal diet produced eggs for a few weeks, but after that laying became intermittent, and finally ceased. The mortality in this group was high, but when birds on the same diet were given 1 gm. of lecithin per day egg production was consistent and mortality decreased.

Rhode Island Red chicks fed the basal diet from the time they were 2 weeks old grew at a normal rate and a few of them came into laying only a little later than the average of the breed raised on the range and fed commercial laying mash.

TABLE 2

Egg production and condition of Rhode Island Red chicks raised on a simplified diet plus choline[1]

NUMBER OF BIRDS	DIET	AVERAGE NUMBER DAYS UNTIL LAYING BEGAN	AVERAGE NUMBER EGGS PER BIRD DURING FIRST 90 DAYS OF LAYING	CONDITION OF BIRDS AT THE END OF 290 DAYS
10	Basal	230	5 (only 4 birds laying)	More than half did not lay at all. Livers very fat and soft. Nearly all the egg yolks aborted.
12	Basal + 25 mg. choline	190	10	In fair condition. Ovaries showed many aborted egg yolks. Livers very soft and fat. Surfaces hemorrhagic.
12	Basal + 50 mg. choline	180	22	In good condition. Few egg yolks aborted. Liver in fair condition.
14	Basal + 75 mg. choline	178	35	In good condition. Only an occasional yolk aborted. Liver in good condition.
6	Basal + 25 mg. choline		(cockerels)	Testes well developed. Sperms alive and numerous. Livers firm and dark. No abdominal fat.
6	Basal		(cockerels)	Testes not so well developed, but sperms were fairly numerous.
7	Laying mash-range chickens	210	30	Liver in good condition.
8	Laying mash-commercial flock	180	35	In good condition. Livers somewhat soft.

[1] Choline added when chicks were 3 months old.

But if, when the birds were 3 months old, 75 mg. of choline were given daily their records in regard to age at which laying began and the number of eggs laid were comparable to those of battery-raised birds in commercial flocks. However, when smaller amounts of choline were fed, egg production was likewise smaller.

The choline-fed birds were killed when they were 290 days old and the following observations made: All the pullets had a considerable store of abdominal fat. This fat was firm and white and resembled mutton tallow. The gizzards were very small and were entirely covered with fat. The proventriculum

TABLE 3
Effect of choline on the fatty acid of chicken liver

SAMPLE NO.	SEX	CHOLINE SUPPLE- MENT	TOTAL FATTY ACID IN LIVER	FATTY ACID IN LIVER	FATTY ACID IN EXTRACT
		mg.	gm.	%	%
1	female	75	0.70	3.13	55.6
2	female	50	3.13	6.67	68.4
3	female	25	6.30	13.00	73.5
4	female	none	8.10	15.70	73.9
5	male	50	0.80	3.19	52.5
6	male	none	2.80	8.22	78.6
7	female	laying mash	4.56	9.71	72.5

was also covered with fat and was abnormal in that it was not constricted at either end. The oviducts and uteri were well formed and appeared normal but the ovaries of the birds fed diets deficient or low in choline showed many aborted egg yolks. Abortion had evidently taken place when the yolks were about two-thirds developed. On the other hand, the ovaries of the birds fed as much as 50 to 75 mg. of choline showed very few aborted yolks.

While the livers of all the pullets were large and soft and light in color, the ones from the birds getting little or no choline were softer and lighter in color than those from birds receiving 50 to 75 mg. of choline. In the former group the

livers showed hemorrhagic surfaces and appeared very fatty. In addition to the effects of choline already noted, the analytical data show that choline inhibits the accumulation of fatty acids in the livers. It will be noted that the concentration of fatty acids in the livers of the males fed diets with or without choline was considerably lower than that in the livers of pullets fed the same rations.

DISCUSSION

In the preliminary experiments the adjustment of the vitamin content of the ration presented the greatest difficulty. Whether the ration as now prepared has an optimum vitamin content awaits further feeding trials. At no time during the experiments did recognizable symptoms of vitamin D deficiency appear. This probably was due to the fact that in addition to the vitamin in the ration the birds were in the sun the greater part of the day. On the other hand, loss of appetite, crop stasis, paralysis and other symptoms indicative of a lack of B_1 or some part of the B_2 complex were common until the daily vitamin intake was 416 Sherman units of B_1 and 326 units of B_2 complex. Even with this intake, occasionally a bird showed symptoms indicative of a lack of some part of the vitamin B complex. Likewise, symptoms of vitamin A deficiency, as evidenced by eye defects and a condition resembling roup were common until 2428 U. S. P. units of vitamin A were given each bird daily.

It is not postulated, however, that the amounts of the various vitamins necessary for growth and reproduction of these birds are the amounts that represent the average requirement of birds on regular poultry rations.

As to the optimum amount of the choline, the other dietary factor, little is known. The data show that when 75 mg. of choline were administered daily, pullets produced more eggs and there were fewer aborted yolks than when 25 to 50 mg. were given. Whether chicks are able to synthesize any or all the lecithin or choline necessary for optimum growth and reproduction is open to question. Best and his collaborators ('34)

are of the opinion that if the rat can synthesize these constituents, the amount is limited. But McCollum, Halpin and Drescher ('12) present data which they interpret as proving "that the synthesis of phosphatides is readily accomplished in the body of the hen when the ration is free from these substances." However, when McCollum's experiment was repeated in this laboratory it was found that, on a diet composed of only rice meal and dried skim milk, 5 to 6 months old pullets laid for a short time, developed symptoms of multiple vitamin deficiency and died. When younger birds were fed the diet, they quickly showed symptoms of vitamin deficiency and did not lay at all. It was evident that stored vitamins and perhaps other constituents necessary for life and reproduction enabled the older birds to lay for a time; but as soon as the stores were exhausted laying stopped and the birds declined, while the younger birds with less stored nutrients died quickly unless the diet was changed.

The data showing that the concentration of fatty acids in the livers of the pullets is greater than that in the livers of the cockerels is of interest. Okey and Yokela ('35) found also that the fatty acid in the livers of female rats fed egg yolks for 120 days was almost three times that of the males. They point out that the fatty acid concentration in the livers of the males decreased with age while that of the females increased. This variation in the accumulation of fatty acids in the livers of male and female rats and in the livers of cockerels and pullets offers interesting possibilities for speculation and suggests some sex variation in the utilization of choline or lecithin. In a preliminary study of a female sex hormone made in this laboratory it was noted that the hormone injected in oil was readily absorbed, but when the oil alone was injected it was very poorly absorbed. Whether the above phenomenon was due to action of the female sex hormone is suggested for consideration. However, in examining livers from non-laying and laying hens and mature cockerels, all fed a commercial feed, differences in size, color and fat content are striking.

Whether choline is a contributing or causative factor is not known.

CONCLUSION

1. The addition of choline to the basal diet increased egg production, decreased mortality, inhibited abortion of egg yolks, and decreased the percentage of fatty acids in the livers.

2. The concentration of fatty acids in livers of the males fed diets with or without choline was considerably lower than that in the livers of pullets fed the same rations.

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QUANTITATIVE REQUIREMENTS OF THE COMPONENTS OF THE VITAMIN B COMPLEX FOR LACTATION AND GROWTH OF NURSING YOUNG OF THE ALBINO RAT[1]

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SIX FIGURES

The investigations of the dietary requirements for fertility and lactation begun in the University of Wisconsin in 1918 and in this laboratory in 1920 were suspended in 1932 awaiting isolation of the various components of the vitamin B complex. It was realized in 1938, of course, that, although B_1 (thiamin), riboflavin, and B_6 have been isolated in pure form as yet unidentified members of the B complex remain to be isolated. However, they may be provided by means of extracts relatively free from known substances. Use of these extracts permits one to construct diets with unknown factors reduced to a minimum. Therefore, lactation studies were resumed last year, and attempts were made to rear nursing young of the albino rat on diets containing the purest available components of the vitamin B complex.

All early attempts to determine the vitamin B_1 requirements for lactation with diets containing autoclaved products, such as Northwestern yeast, autoclaved beef, and autoclaved liver, resulted in failure in most trials. As much as 15% autoclaved liver and 10% autoclaved beef did not always insure normal

rearing of young, even when supplemented with 60 µg. of thiamin daily. It became apparent that considerable destruction of components of the vitamin B complex, other than B_1, occurs during autoclaving, and in order to satisfy the requirements of such dietary essentials for lactation, the procedure of using natural foods cooked under pressure had to be abandoned.

When an attempt was made to prepare the synthetic vitamin B complex mixture, vitamin B_1[2] and riboflavin[3] were available in pure crystalline form. Vitamin B_6 had just been isolated by Lepkovsky ('38) and independently by Keresztesy and Stevens ('38), and by György ('38). György and co-workers had previously reported ('37) that Peter's eluate, free from riboflavin, was a dependable source of vitamin B_6, 1 cc. being equivalent to 10 gm. of yeast, and that this eluate contained an additional substance, termed the 'maturation factor.' A deficiency of the 'maturation factor', according to György and associates, produced panmyelophthisis in the rat ('37). Since the procedure employed for the preparation of 'B_6 and the maturation factor' was the one originally used by Kinnersley and associates ('33) for the production of B_4, it was considered that, if B_4 is essential for the rat as claimed by Kline and associates ('36), it would be provided by Peter's eluate.

It soon became evident, however, that the 'B_6 and maturation factor' extracts were not a dependable source of B_6 for lactation purposes, and furthermore, they were much too expensive to permit numerous experiments. A continuation of the work was then made possible by generous amounts of B_6 crystals furnished by Dr. S. Lepkovsky. Since the chick antidermatitis factor of Lepkovsky and co-workers ('36) had not been established as a dietary essential for the rat, it was not used. The Peter's eluate (B_6 and maturation factor) furnished a component other than thiamin, riboflavin, B_6, choline and nicotinic acid. This component, however, was found in a much more concentrated form in preparations designed to

[2] Generously supplied by Merck and Company.
[3] Generously supplied by Hoffman-LaRoche Company.

furnish the 'W' factor of Elvehjem, Koehn, and Oleson ('36). Choline was used in the synthetic vitamin mixture, since McHenry ('35), and Best and associates ('36) have reported its presence to influence the growth of rats under certain dietary restrictions. Although nicotinic acid, found to be essential for the prevention and cure of black tongue in dogs (Elvehjem, Madden, Strong and Woolley, '38), and to be the human antipellagric factor (Smith and associates, '37; Spies and co-workers, '38; Bogart, '38) had not yet been shown to be essential for the rat, it was incorporated in the vitamin mixture, in order to be sure that it would not be a limiting factor. The nursing mothers received 6 mg. nicotinic acid daily.

In order positively to satisfy the protein requirements for lactation, the proportion of casein in the ration was increased from 20% (used in earlier work) to 25% supplemented with 0.2 to 0.5% cystine (see rations 6 and 7, table 1). This procedure was suggested by the work of Daggs and Lidfeldt ('38). When it became apparent by the losses of weight of the mother and litter that a dangerous period of depletion of vitamin reserves had been reached,[4] supplements of the various components of the vitamin B complex were given daily separately from the ration. Ten drops a day of cod liver oil[5] supplied vitamins A and D. Choline was furnished in the form of the chloride, 15 mg. being the dose used daily.[6]

Space does not permit the report here of our extensive data. Typical lactation records, representing about one-sixth of the total data, are submitted graphically in figures 1 to 6 inclusive. The composition of rations is given in table 1. Growth records of mothers and litters were taken daily. Food consumption records were also taken daily, but are not reported here in order to conserve space.

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[4] The dietary technic employed was that outlined in previous publications (Sure, '28 a, b, c).

[5] Obtained from Mead Johnson and Company.

[6] Obtained from The Eastman Kodak Company.

Lactation efficiency on stock diet no. 1

Stock diet no. 1 is a cereal ration containing 1% cod liver oil, 5% commercial casein, 0.5% NaCl and 0.5% CaCO₃. It is supplemented 6 days a week with 5 cc. fresh cow's milk per animal until breeding, when the amount is increased to 10 cc., and during lactation, when the young begin eating, to 15 cc. daily. Each animal also received 15 gm. fresh lettuce once weekly. On such a dietary regime about 95% of the young are raised. During the nursing period the mother either main-

TABLE 1

Composition of rations

	No. 6	No. 7	No. 8
Casein[1]	25.0	25.0	25.0
Agar-agar	2.0	2.0	
Salts 185[2]			4.0
Salts 351[3]	4.0	4.0	
Cystine	0.2	0.5	
Brewer's yeast			2.0
Butterfat	10.0	10.0	10.0
Dextrin	58.8	58.5	44.0
Liver (autoclaved 6 hrs. at 15 lbs. pressure)			15.0

[1] Extracted several times with hot 95% alcohol and cold 25% alcohol.

[2] McCollum, E. V., and Nina Simmonds 1918 A study of the dietary essential, water-soluble B, in relation to solubility and stability towards reagents. J. Biol. Chem., vol. 33, p. 63.

[3] Hubbell, Rebecca B., Lafayette B. Mendel and Alfred J. Wakeman 1937 A new salt mixture in experimental diets. J. Nutrition, vol. 14, pp. 273–285.

tains her weight or may gain 8 to 10% of the body weight at parturition. The young open their eyes about the fifteenth day and begin eating of the maternal diet between the seventeenth and twentieth day. The litter is considered weaned when each young rat weighs 40 gm. which generally takes place the twenty-second to the twenty-fifth day of lactation (fig. 1). The dotted line curve in the figures submitted indicates the body weight of the nursing mother whereas the heavy line curve denotes the growth of the litter. In determining the optimum amount of any of the components of the vitamin B complex

essential for lactation, or in the consideration of the need of additional vitamin essentials for milk secretion, the lactation efficiency index on stock diet no. 1 was used as a basis for comparison. Appropriate reference curves indicating this standard performance are shown in the respective figures.
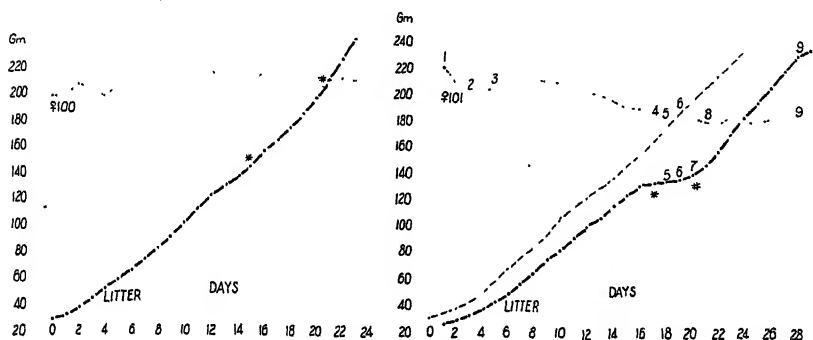
Fig. 1   Lactation record of ♀100 (159 days old) on stock diet no. 1. *, young opened their eyes. #, young eating.

Fig. 2   Lactation record of ♀101 (171 days old). Showing vitamin B_1 requirements for lactation and for growth of nursing young. 1, ration no. 6; 2, ration no. 8; 3, ration no. 7, supplemented with 120 µg. riboflavin, 50 µg. vitamin B_6, 6 mg. nicotinic acid, 15 mg. choline, and 1 cc. W factor to mother; 4, 60 µg. B_1 to mother; 5, 18 µg. B_1 to mother, and 42 µg. B_1 to litter; 6, 18 µg. B_1 to mother. and 60 µg. B_1 to litter; 7, 90 µg. B_1 to litter; 8, 30 µg B_1 to mother; 9, remove all vitamin supplements from mother and give litter 120 µg B_1. *, young opened their eyes. #, young eating. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

Thiamin and riboflavin requirements for lactation

In chronic vitamin B_1 deficiency produced on rations containing autoclaved yeast and beef (Sure, '38) 10 µg. of thiamin[7] was always sufficient for the cure of polyneuritis associated with marked convulsions, and was always accompanied by continuous and satisfactory growth. In attempting to determine the thiamin requirement for lactation on rations containing 25% autoclaved yeast and beef or autoclaved liver and beef, as much as 60 µg. B_1 did not definitely assure normal rearing of the young. After the completion of numerous ex-

[7] Thiamin and B_1 are used interchangeably in this paper.

periments it became evident that 120 μg. of thiamin had to be included in the synthetic mixture of the components of the vitamin B complex to prevent polyneuritis in the young and to insure weaning of the litter at a rate comparable to that secured with our stock diet no. 1 (Sure, '26).

After it became evident that, following depletion of the reserves of the vitamin B complex, 120 μg. B_1, 120 μg. riboflavin, 50 μg. B_6, 15 mg. choline chloride, 1 cc. W^8 factor, and 6 mg. nicotinic acid would permit rearing of the young at a rate very nearly comparable to that secured on our stock diet no. 1, it was then possible to vary quantitatively any of the constituents in this mixture of the vitamin components.

From figure 2 it is clear, judging by the character of growth of the litter of ♀ 101, that 60 μg. B_1 furnished the mother or distributed between mother and young on the seventeenth to the twentieth day of lactation, was inadequate for the continuous growth of the nursing young. Desired growth was accomplished only after the litter received 90 μg. B_1 and the mother 30 μg. B_1. In numerous other experiments litters were reared on a daily dose of 120 μg. B_1 given to the mother but in many instances the vitamin had to be distributed between young and mother, allowing about three-fourths of the daily dose to the litter and one-fourth to the mother. This offers corroborative evidence of former experiments (Sure, '28a) of the inefficiency of the lactating mother in transferring vitamin B_1 to the milk. Such a phenomenon will become more apparent from an analysis of the other figures. That such inefficiency is not confined exclusively to thiamin will also become evident from an examination of the rest of the data.

Figure 3 shows that growth of the litter of ♀ 102 was tremendously retarded until the daily dose to the mother was increased to 90 μg. riboflavin.

From figure 4 it is evident that, whereas 90 μg. riboflavin failed when given to the mother, a distribution of 30 μg. to the mother and 60 μg. to the litter yields successful results. The

[8] Equivalent to 1 gm. of Wilson's liver extract.

first plateau in the curve of growth of the litter was reached between the twenty-seventh and thirtieth day of lactation because of riboflavin deficiency. The second plateau appeared on the thirty-fifth day when increased allowance of riboflavin to the litter brought no response but a distribution of vitamin B_1 between mother and young resulted in increased growth.
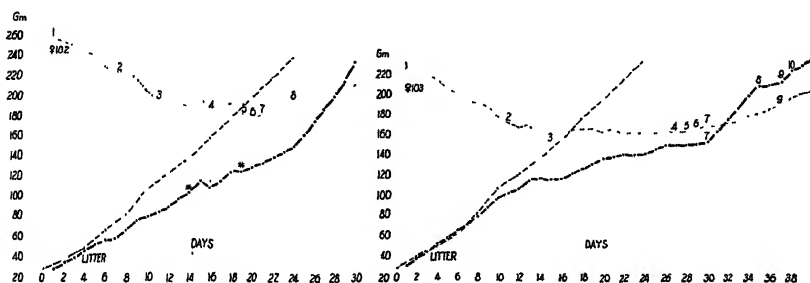
Fig. 3   Lactation record of ♀102 (191 days old). Showing riboflavin requirements for lactation. 1 Ration no. 7. 2 120 μg. B_1, 50 μg. vitamin B_6, 6 mg. nicotinic acid, 15 mg. choline, and 1 cc. W factor to mother. 3 10 μg. riboflavin to mother. 4 20 μg. riboflavin to mother. 5 30 μg. riboflavin to mother. 6 40 μg. riboflavin to mother. 7 60 μg. riboflavin to mother. 8 90 μg. riboflavin to mother. * Young opened their eyes. # Young eating. Curve in thin line represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

Fig. 4   Lactation record of ♀103 (167 days old). Showing riboflavin requirements for lactation and for growth of nursing young. 1 Ration no. 7. 2 120 μg. B_1, 50 μg. vitamin B_6, 15 mg. choline, 6 mg. nicotinic acid, and 1 cc. W factor to mother. 3 20 μg. riboflavin to mother. 4 30 μg. riboflavin to mother. 5 60 μg. riboflavin to mother. 6 90 μg. riboflavin to mother. 7 30 μg. riboflavin to mother, and 60 μg. riboflavin to litter. 8 90 μg. riboflavin to litter. 9 30 μg. B_1 to mother, and 90 μg. B_1 to litter. 10 Remove B_1 from mother and give 120 μg. B_1 to litter. * Young opened their eyes. # Young eating. § Alopecia in young. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

Vitamin B_6 requirements for lactation

From the results of eight lactation experiments it became evident that it is necessary to introduce at least 50 μg. of B_6 for lactation, in order to rear young at a rate approximating that obtained on stock diet no. 1 (fig. 1). For continuous growth 10 to 25 μg. B_6 were found adequate. To conserve space, however, such data are not presented here.

The 'W'[9] factor versus 'B₆ and maturation factor' for lactation

A biological assay of the preparation used to supply the 'W' factor for various components of the vitamin B complex disclosed that it contained small amounts of vitamin B_6 for growth but negligible amounts for lactation. It was found to be practically free from thiamin and riboflavin.

In the early trials an attempt to rear young of the albino rat with supplements of thiamin, riboflavin, vitamin B_6, choline, and nicotinic acid resulted in complete failure about the tenth to fifteenth day of lactation. The addition of 3 cc. of the 'B_6 and maturation factor' extract[10] resulted in the rearing of three litters out of twelve. This extract must have furnished some component or components other than thiamin, riboflavin, B_6, choline, or nicotinic acid. It was then decided to introduce the 'W' factor of Elvehjem and associates ('36, '37). This resulted in success in every experimental trial. It was found that 1 cc. of the solution containing the 'W' factor was many times more potent than 3 cc. of that which furnished the 'B_6 and maturation factor.' This is brought out in figure 5 (see eighteenth day). The accelerated growth of the litter after the daily dose of thiamin was increased to 120 µg. is also apparent.

From figure 6 it is apparent that lactation proceeds at a rate comparable to that secured on stock diet no. 1 when the synthetic mixture of the components of the vitamin B complex given the nursing mother contains 120 µg. B_1, 120 µg. riboflavin, 50 µg. B_6, 15 mg. choline chloride, 6 mg. nicotinic acid, and 1 cc. of a solution containing 'W' factor, equivalent to 1 gm. of liver extract. The litter of ♀105, which was reared in 24 days, would have been weaned sooner if there had not been

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[9] The 'W' factor was prepared from liver extracts furnished by Dr. David Klein of the Wilson Laboratories, according to the technic of Frost and Elvehjem ('37).

[10] Furnished by the S.M.A. Corporation. The 3 cc. of extract are equivalent to 30 gm. yeast, prepared according to the method of Kinnersley and associates ('33) and referred to in the literature as Peter's eluate.

a depletion period of 4 days on the maternal diet in this experiment. One change was made in this instance, namely, on the seventeenth day of lactation when the young were growing at a subnormal rate, the 120 µg. dose of B_1 was distributed between mother and young, one-fourth to the former and three-fourths to the latter. Following this change the litter exhibited accelerated growth.
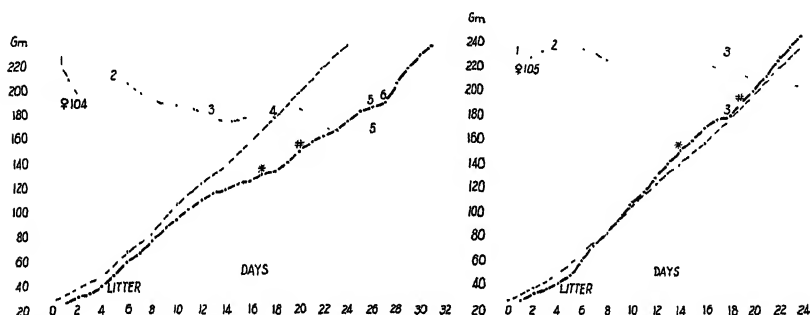
Fig. 5 Lactation record of ♀104 (120 days old). Showing the potency of the W factor versus the B_6 and maturation factor extract for lactation; also the inefficiency of this mother in secreting B_1 in the milk. 1 Ration no. 7. 2 120 µg. B_1, 120 µg. riboflavin, 50 µg. crystalline B_6, 15 mg. choline, 6 mg. nicotinic acid to mother. 3 3 cc. B_6 and maturation factor extract to mother. 4 Replace the 3 cc. B_6 and maturation factor extract with 1 cc. W factor extract. 5 30 µg. B_1 to mother, and 90 µg. B_1 to the litter. 6 Increase to 120 µg. B_1 to the litter. * Young opened their eyes. # Young eating. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

Fig. 6 Lactation record of ♀105 (256 days old). Showing that lactation proceeds at a rate comparable with that obtained on stock diet no. 1 (fig. 1) on a synthetic mixture of the vitamin B complex when adequate amounts of the various components are furnished as outlined below. 1 Ration no. 6. 2 Ration no. 7, supplemented with: 120 µg. B_1, 120 µg. riboflavin, 50 µg. B_6, 6 mg. nicotinic acid, 15 mg. choline, and 1 cc. W to mother. 3 30 µg. B_1 to mother and 90 µg. B_1 to litter. * Young opened their eyes. # Young eating. Curve in thin lines represents growth of litter on stock diet no. 1 which represents the normal curve of growth.

DISCUSSION

The results of the studies reported in this paper deserve some comment. As far as the author is aware, these are the first lactation investigations carried out with the use of synthetic diets free from autoclaved natural foods. Four vitamins, which are recognized as dietary essentials for the rat,

dog and man, namely, thiamin, riboflavin, B_6, and nicotinic acid, have been used in pure form. In addition, a fifth product, choline chloride, has been included in the synthetic mixture of the components of the vitamin B complex, which must now be recognized as essential for the rat (Sure, '40).

Since the completion of the work reported here, six litters have been successfully reared without nicotinic acid administered either to the mother or to the young. Neither could it be demonstrated in this laboratory that nicotinic acid is essential for the weaned rat subsisting on highly purified diets. Our results are in accord with the recent findings of Birch ('39). Frost and Elvehjem ('39) however, reported that they found nicotinic acid to be essential for the rat when they introduced 12% white corn in their rations and interpreted this to mean that the corn furnishes a factor necessary for the utilization of nicotinic acid.

Several litters have also been reared on our synthetic ration containing 25% casein without cystine additions. This high per cent of casein probably furnishes enough methionine to make it a complete protein for lactation.

If there exist needed components of the vitamin B complex other than thiamin, riboflavin, B_6, and choline, these unidentified substances must have been furnished by the solution containing 'W' factor, which was prepared from liver extracts (Wilson). For example, the spectacled appearance observed by Oleson and associates ('39), the panmyelophthisis reported by György and co-workers ('37) and the B_4 deficiency reported by Kline and associates ('36), have not been observed in the nursing young in this investigation. Perhaps the symptomatology nearest to that of B_4 deficiency observed here was the paralysis in nursing young encountered in choline deficiency. In this connection it may be mentioned that McHenry ('35) considered the symptoms exhibited by his choline deficient rats to resemble those of B_4 deficiency as described by Reader ('29, '30 a, b).

The much greater requirement of thiamin, riboflavin, and B_6 for lactation may perhaps be best explained as due to the

utilization of greater amounts of food ingested during this period. This explanation, however, is not entirely satisfactory, because, although the food intake during lactation is doubled or trebled, the thiamin and riboflavin requirements for rearing of young are at least five·to six times that required for growth on highly purified diets free from autoclaved foods and ten to twenty.times that necessary- for the cure of such avitaminotic symptoms as polyneuritis and cataracts. Enough vitamin must be supplied not for one individual but for the mother and the rapid development of six individuals, since, according to the method, each lactating mother is allowed six young to rear.

Another puzzling problem that awaits solution is the difficulty many mother rats have in transferring to the milk the pure thiamin, riboflavin, B_6, and choline chloride, a phenomenon not observed when satisfactory diets are composed of natural foods. It may be of interest to relate here an experience noted in several lactation experiments. After the publication of the recent report of Perla ('39) that manganous chloride exerts a protective action against injurious effects of massive doses of vitamin B_1 on lactation, this salt was given to mother rats which were inefficient in transferring thiamin to the milk. In three instances a daily dose of 2 mg. manganous chloride permitted the mothers to rear their litters without the necessity of distribution of the vitamin between mother and young.

SUMMARY

1. A study has been made of the amounts of various components of the vitamin B complex needed for successful lactation.

2. Following depletion of vitamin reserves, it has been determined that rearing of the young of the albino rat can proceed at a rate comparable with that secured on an optimum stock diet when the following supplements are furnished daily to the mother: 120 µg. B_1 (thiamin), 120 µg. riboflavin, 50 µg. B_6, 15 mg. choline chloride, 1 cc. of a solution of 'W' factor

equivalent to 1 gm. of liver extract, and 6 mg. nicotinic acid.
It was not determined whether the nicotinic acid is really es-
sential for the lactating rat.

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THE ESSENTIAL NATURE OF CHOLINE FOR LACTATION AND GROWTH OF THE ALBINO RAT[1]

BARNETT SURE

WITH THE TECHNICAL ASSISTANCE OF ALETHEA BEACH

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THREE FIGURES

The lipotropic action of choline has been demonstrated by Best and co-workers ('34, '35 and '36) and confirmed by Perlman and Chaikoff ('39). McHenry ('35) and Best and associates ('36) have also produced evidence that choline influences the growth of rats. The latter workers emphasized that, in order to demonstrate effects of choline deficiency the diet must contain a high proportion of fat (40%) and a low concentration of protein, if casein is the source of nitrogen in the ration because of the lipotropic effect of that protein. The average period of experimental study was about 21 days. The results of the work of these investigators suggested the introduction of choline into the mixture intended to supply the components of the vitamin B complex. It was decided to test the effect of the daily administration of 15 mg. choline chloride during lactation. This was found satisfactory in most instances. In several cases rearing of the young to weaning was accomplished only after this dose was divided between mother and young. The diet had the following composition: casein (extracted several times with hot 95% alcohol) 25%; agar-agar 2.0; salt mixture 4 [2]; cystine 0.5; butterfat 10; dextrin 58.5%.

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[1] Research paper no. 623, journal series, University of Arkansas. This is paper XXVII in the series of Dietary Requirements for Fertility and Lactation.

[2] Hubbell, Rebecca B., Lafayette B. Mendel and Alfred J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, pp. 273–285.

In addition each mother received daily 120 µg. thamin, 120 µg. riboflavin, 6 mg. nicotinic acid, and 1 cc. of a solution containing the 'W' factor equivalent to 1 gm. liver extract (Wilson).

In figures 1 and 2 are presented lactation records typical of those yielded by six mothers. They show that choline is a dietary essential for the growth of nursing young of the albino rat. Figure 1 is a lactation record showing depletion of choline on the thirteenth day and the response obtained in the growth of the litter following an administration of 15 mg. choline chloride daily. Before the choline was given the young developed paralysis similar in symptomatology to that described by McHenry in weaned rats ('35) and Reader ('29
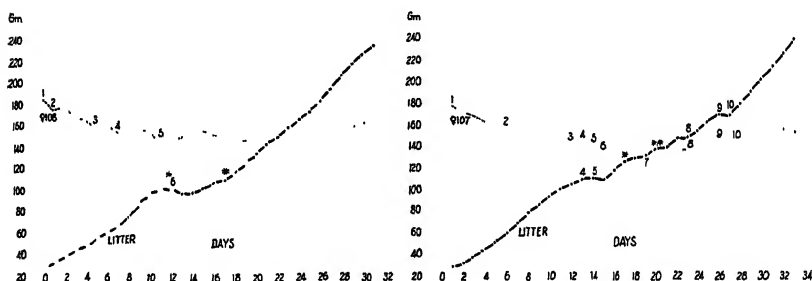
Fig. 1   Lactation record of ♀108 (85 days old). Showing choline to be an essential dietary factor for growth of nursing young of the albino rat. 1, stock diet no. 1; 2, ration no. 22,[1] 3, ration no. 7; 4, 120 µg. B_1, 120 µg. riboflavin, 25 µg. B_6, and 0.5 cc. W factor to mother; 5, 50 µg. B_6 and 1 cc. W factor to mother; 6, 15 mg. choline to litter. *, young opened their eyes. #, young eating.

Fig. 2   Lactation record of ♀107 (155 days old). Showing that choline is an essential component of the vitamin B complex for growth of nursing young of the albino rat; also that this mother was inefficient in secreting this dietary essential in the milk. 1, ration no. 7; 2, 120 µg. B_1, 120 µg. riboflavin, 50 µg. B_6, 15 mg. choline to mother; 3, 6 mg. nicotinic acid to mother; 4, 30 µg. B_1 to mother and 90 µg. B_1 to litter; 5, 30 µg. riboflavin to mother, and 90 µg. riboflavin to litter; 6, 3 cc. B_6 and maturation factor extract to mother; 7, replace the 3 cc. B_6 and maturation factor with 1 cc. W factor to mother; 8, 20 µg. crystalline B_6 to mother and 30 µg. crystalline B_6 to litter; 9, 10 µg. B_6 to mother and 40 µg. B_6 to litter; 10, 5 mg. choline to mother, and 10 mg. choline to litter. *, young opened their eyes. **, young eating.

[1] Composition of ration no. 22 is as follows: Casein (extracted with hot alcohol) 25; agar-agar, 2; salts 351, 4; Crisco, 10; dextrin, 59.0.

and '30). The paralysis disappeared the day following the administration of choline. Apparently the mother did not need choline, because she maintained her body weight from the time the choline therapy to the litter was begun.

From figure 2 it is evident that, although the litter of ♀ 107 began partaking of the maternal diet the twentieth day of lactation, satisfactory growth of the young was not secured until the daily dose of 15 mg. choline chloride was distributed between mother and young on the twenty-seventh day of lactation, allowing 5 mg. to the former and 10 mg. to the latter.

Although the choline deficiency in the nursing young of the rat in this study was produced on a ration containing 10% fat, it should be taken into consideration that the milk of the rat contains 32% fat (Donaldson, '24). Therefore, the nursing young were on essentially a high-fat diet.

In a number of cases choline chloride was withheld from the vitamin B complex mixture, and this resulted in the death of the litters about the fifteenth to the eighteenth day of lactation. The avitaminotic state in the nursing young of the rat is characterized chiefly by paralytic symptoms indistinguishable from B_1 polyneuritis in litters of the same age, but without associated running screaming fits (Sure, '28).

Following the accumulation of evidence that choline is an indispensable dietary factor for the growth of nursing young, attempts were made to determine whether choline is essential for growth of the weaned rat, employing our own dietary technique. McHenry's work ('35) may be criticized on the ground that vitamin B_1 is the only constituent he introduced to satisfy the requirements of the vitamin B complex. Furthermore, neither McHenry nor Best and associates ('36) established that their synthetic rations contained sufficient riboflavin or B_1. In the construction of our purified diets it was felt desirable to avoid the introduction of a natural food such as egg-white. The early experiments, involving 116 animals fed rations consisting of 20% purified casein and 10% fat, yielded inconsistent results. After some correspondence with Doctor Best, it was decided to use a high-fat low-protein

diet, and a ration was constructed, on which choline deficiency has been produced in twenty-four groups of animals. The composition of the diet was as follows: fibrin (extracted with hot alcohol) 9%; agar-agar 2; salt mixture 4; Crisco 30; and
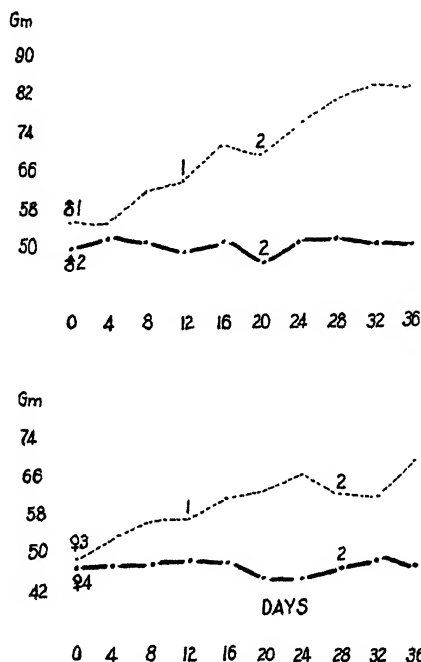
Fig. 3 Showing the influence of choline on growth of weaned rats on a diet containing 9% fibrin and 30% fat (Crisco). Straight line curves represent the growth of the animals on a diet deficient in choline. Dotted line curves represent the growth of litter mates of the same sex which were restricted to the same amount of food consumed by the choline-deficient rats, but which in addition received 6 to 15 mg. choline chloride daily.

[1] Increased choline chloride from 6 to 15 mg. daily.
[2] One-tenth cubic centimeter W factor daily (equivalent to 0.1 gm. liver extracts).

sucrose (extracted with hot alcohol) 55.0%. The majority of the experiments were conducted by the paired feeding method, the control litter mate animals of the same sex being restricted daily to the amount of food consumed by those receiving the choline-deficient diet. Each animal received daily separately

from the ration 20 µg. thiamin, 20 µg. riboflavin, and 10 µg. B_6. The control received in addition 6 to 15 mg. daily of choline chloride. Two typical cases are presented in figure 3. Several of the choline deficient animals which died were found at autopsy to have hemorrhages in the cortex of the kidneys which is in agreement with the recent findings of Griffith and Wade ('39). After about 4 to 6 weeks of growth, the control as well as the choline-deficient animals fail to gain because of the deficiency of other components of the vitamin B complex. The supplementation with 0.1 cc. to 0.2 cc. of the solution containing the 'W' factor brings a response, but often the choline-deficient animal also resumes growth. This probably means that the solution used to supply factor 'W' also furnished the lipotropic factor, choline chloride or some other substance. We are observing at this time that the change from purified sucrose to dextrin exerts a stimulating effect on growth, as recently reported by Oleson and associates ('39).

SUMMARY

Choline has been found to be an indispensable component of the vitamin B complex for growth and lactation of the albino rat.

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THE INFLUENCE OF THE NITROGEN CONTENT OF THE DIET ON THE CALORIE BALANCES OF PRE-SCHOOL CHILDREN [1]

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ONE FIGURE

Although there have been many studies showing the calorie needs of pre-school children, there have been few balance studies which determined both the actual number of calories obtained from food and those lost in the excreta. Such studies give accurate values for available calories and show the influence of various factors upon availability.

In connection with their studies on the metabolism of undernourished children, Wang and her associates ('28 a, '28 b and '29) determined 3-day calorie balances. They calculated the intake values (Rose, '25), analyzed the urine and feces by means of the Bomb calorimeter and determined the available calories by difference. Although values for calorie intake calculated by means of physiological values from which excretory losses had been subtracted may vary considerably from analyzed figures (Donelson and her associates, '31; Bray, Hawks and Dye, '34), the results of Wang and her associates indicate several interesting trends. All of the

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[2] Some of the data reported in this paper were presented by Jeanne M. Voorhees in partial fulfillment of the requirements for the degree of master of science, Michigan State College.

[3] Some of these data were presented before the American Home Economics Association, Chicago, Illinois, June, 1935.

children they studied, both those normal in weight and undernourished, absorbed and retained approximately the same percentage of the calorie intake, indicating that the calorie loss for 24 hours, both in urine and feces, increased with intake. This constant percentage utilization was the same whether the children did or did not gain in weight. At the same time the results suggest that there may be a relationship between the protein and calorie contents of the diet, because, when the protein content was increased the results showed an increase in eliminated calories, both in urine and feces. In view of these findings, it would seem that balance studies, conducted over long periods of time and which gave actual values, might produce further information on the availability of calories and the effect of diet on calorie balance.

The purposes of the present study were to determine whether small period-to-period variations in intake calories influence their availability and to ascertain the effect on calorie balance of increasing the protein content of the diet. Before these purposes could be accomplished, however, it was necessary to determine the length of time required for a preliminary period, especially following a change in the protein content of the diet and also to learn whether slight differences in the composition of diets would be sufficient to cause variations in results.

EXPERIMENTAL PROCEDURE

The data on calorie balances given in this paper, as well as those for nitrogen utilization discussed in a previous paper (Hawks, Bray and Dye, '38) were obtained during two long-continued balance studies on five pre-school children. For the sake of brevity, the condition and care of the children, preparation and composition of the diets and all details of experimental procedure, which were given in the first report of the two experiments (Hawks, Dye and Bray, '37) are omitted. The children, however, lived under extremely constant conditions for relatively long periods of time. The two children in the first experiment did not have exactly the same foods

as the three children in the second experiment, but they all had, first, a diet containing 3 and then one containing 4 gm. of protein per kilogram per day. All technical procedures were exactly the same. The calorie values of food, urine and feces, for 3-day collection periods, were determined by means of the oxy-calorimeter (Benedict and Fox, '25 a and '25 b).

RESULTS AND DISCUSSION

In order to interpret the results of an experiment, it is important to know when the subjects reach an equilibrium and how much of the experimental data should be considered as preliminary. Therefore it was necessary to determine, first, whether the children were in calorie equilibrium when receiving the 3-gm. protein diet and second, the length of time necessary to re-establish equilibrium following the change to the 4-gm. protein diet. The data for the seven 3-day periods on the medium protein diet (table 1) collected following preliminary periods of 10 or 12 days, were extremely constant and showed that the children were undoubtedly in calorie equilibrium. The small period-to-period fluctuations in intake had little effect on urine and feces values, since, with the exception of the feces values for the children in the first experiment, all output figures varied within one calorie for each child and represented a relatively constant percentage of the intake calories. Statistical evaluation of the data showed coefficients of variation for urine and feces ranging from 5.6 to 12.8. Although relatively larger than those for intake, which were 1.2 and 1.3, they were not high. The absorption and retention values also indicated that the children were in calorie equilibrium. The coefficients of variation for absorption, retention and intake were practically identical for each experiment, being 1.5, 1.5 and 1.2, respectively, in experiment 1, and 3.5, 3.6 and 3.3 in experiment 2. These similar coefficients showed that absorption and retention values fluctuated to the same degree as intake figures. The high correlations between absorption and intake, 0.746 and 0.939 for experiment 1 and 2, respectively, and between retention and intake, 0.731 and

TABLE 1

Calorie balance data on a constant 3-gm. protein diet, expressed per kilogram of body weight

CHILD	EXPERIMENT	PERIOD	INTAKE CALORIES	OUTPUT							ABSORPTION		RETENTION	
				Urine			Feces		Total					
				Total calories	Proportion intake	Ratio calories/ nitrogen	Total calories	Proportion intake	Total calories	Proportion intake	Total calories	Proportion intake	Total calories	Proportion intake
					%			%		%		%		%
B	I	1	85.8	3.4	4.0	8.8	6.1	7.1	9.5	11.1	79.7	92.9	76.3	88.9
		2	87.0	3.1	3.6	8.2	6.6	7.6	9.7	11.2	80.4	92.4	77.3	88.8
		3	85.8	3.1	3.6	7.8	5.2	6.1	8.3	9.7	80.6	93.9	77.5	90.3
		4	85.1	3.3	3.8	7.8	4.9	5.8	8.2	9.6	80.2	94.2	76.9	90.4
		5	86.4	3.2	3.7	8.0	5.3	6.1	8.5	9.8	81.1	93.9	77.9	90.2
		6	86.8	3.5	4.1	8.5	5.1	5.8	8.6	9.9	81.7	94.2	78.2	90.1
		7	87.4	3.4	3.9	8.2	4.9	5.6	8.3	9.5	82.5	94.4	79.1	90.5
		Mean	86.3	3.3	3.8	8.2	5.4	6.3	8.7	10.1	80.9	93.7	77.6	89.9
D	I	1	84.9	3.4	4.0	8.2	6.9	8.1	10.3	12.1	78.0	91.9	74.6	87.9
		2	86.5	3.2	3.7	7.7	5.8	6.7	9.0	10.4	80.7	93.3	77.5	89.6
		3	85.0	3.1	3.7	7.2	4.5	5.3	7.6	9.0	80.5	94.7	77.4	91.0
		4	83.9	3.1	3.8	7.3	4.9	5.8	8.0	9.6	79.0	94.0	75.9	90.4
		5	85.0	3.1	3.7	7.3	5.8	6.8	8.9	10.5	79.2	93.2	76.1	89.5
		6	86.0	3.1	3.6	7.0	5.2	6.0	8.3	9.6	80.8	94.0	77.7	90.4
		7	86.8	2.8	3.2	6.7	5.4	6.2	8.2	9.4	81.4	93.8	78.6	90.6
		Mean	85.4	3.1	3.6	7.3	5.5	6.5	8.6	10.1	79.9	93.5	76.8	89.9
V	II	1	90.7	2.4	2.6	6.0	3.7	4.1	6.1	6.7	87.0	95.9	84.6	93.3
		2	87.0	2.8	3.1	6.9	3.7	4.3	6.5	7.4	83.3	95.7	80.5	92.6
		3	—	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	—	—	—	—	—	—	—
		5	87.1	2.7	3.1	7.0	4.0	4.6	6.7	7.7	83.1	95.4	80.4	92.3
		6	88.1	2.8	3.2	7.0	3.7	4.2	6.5	7.4	84.4	95.8	81.6	92.6
		7	86.6	2.6	3.0	6.9	3.6	4.2	6.2	7.2	83.0	95.8	80.4	92.8
		Mean	87.9	2.7	3.1	6.8	3.7	4.2	6.4	7.3	84.2	95.8	81.5	92.7
C	II	1	94.0	2.8	3.0	7.0	4.4	4.7	7.2	7.7	89.6	95.3	86.8	92.3
		2	90.6	2.6	2.9	6.6	4.5	5.0	7.1	7.9	86.1	95.0	83.5	92.1
		3	91.6	2.8	3.1	6.9	4.4	4.8	7.2	7.9	87.1	95.2	84.4	92.1
		4	92.2	3.1	3.4	7.6	4.4	4.8	7.5	8.2	87.8	95.2	84.7	91.8
		5	90.4	3.0	3.3	7.6	4.5	5.0	7.5	8.3	85.9	95.0	82.9	91.7
		6	91.5	2.8	3.0	6.9	4.3	4.7	7.1	7.7	87.2	95.3	84.4	92.3
		7	89.8	2.8	3.1	7.4	4.2	4.7	7.0	7.8	85.6	95.3	82.8	92.2
		Mean	91.4	2.8	3.1	7.1	4.4	4.8	7.2	7.9	87.0	95.2	84.2	92.1
J	II	1	88.0	2.6	2.9	7.2	4.2	4.8	6.8	7.7	83.8	95.2	81.3	92.3
		2	84.6	2.5	3.0	6.9	4.6	5.4	7.1	8.4	80.0	94.6	77.5	91.6
		3	85.5	2.6	3.1	7.2	4.6	5.4	7.2	8.5	80.9	94.6	78.3	91.5
		4	86.8	2.7	3.1	7.3	4.9	5.6	7.6	8.7	81.9	94.4	79.2	91.3
		5	85.1	2.7	3.2	7.3	4.9	5.8	7.6	9.0	80.2	94.2	77.5	91.0
		6	85.9	2.7	3.1	7.3	4.5	5.2	7.2	8.3	81.4	94.8	78.7	91.7
		7	84.1	2.7	3.2	7.8	4.5	5.3	7.2	8.5	79.6	94.7	76.9	91.5
		Mean	85.7	2.6	3.1	7.3	4.6	5.4	7.2	8.4	81.1	94.6	78.5	91.6

0.936 in the two experiments, as well as the constant percentages of the intake absorbed and retained (table 1) indicated that all of these values fluctuated in the same manner.

The change to the 4-gm. protein diets, which immediately followed use of the medium protein diets, did not disturb the calorie equilibrium (table 2). In the study of nitrogen balances, Hawks, Bray and Dye ('38) found that the values fluctuated more during the first periods following the change in diets than in succeeding periods, and in the study of the variation in the urinary nitrogenous constituents ('37), they reported more variable data during the first three periods on the high protein diets. In the present study on calorie balances there seemed to be no consistent differences between the values for the first periods following the change in diets and those for the subsequent periods, and there were no significant differences between the mean values of the data for all of the periods together, for the first three periods or for periods four to eight. Statistical evaluation of the data also showed that there was no need for a preliminary period. Both the standard deviations and the coefficients of variation for all output figures and for absorption and retention values were practically the same for the last five periods and for the total values representing all periods. Although preliminary periods may not have been necessary in this study, it does not follow that they could be omitted from all calorie balance studies. If the calories or other factors varied to a great extent, preliminary periods might be necessary.

Although the diets used in the two experiments were practically the same, there were some differences in the results, which can be seen by comparing the mean values of the results obtained in the first experiment with those obtained in the second. Table 1 shows that the percentages of the intake excreted and retained were almost identical for the children within each experiment, but that the results for the two experiments differed. For example, the two children in the first experiment excreted identical percentages of the calorie intake, but a larger percentage than the children in the second

TABLE 2

Calorie balance data on a constant 4-gm. protein diet, expressed per kilogram of body weight

CHILD	EXPERIMENT	PERIOD	INTAKE CALORIES	OUTPUT							ABSORPTION		RETENTION	
				Urine			Feces		Total					
				Total calories	Pro-portion intake	Ratio calories/ nitrogen	Total calories	Pro-portion intake	Total calories	Pro-portion intake	Total calories	Pro-portion intake	Total calories	Pro-portion intake
					%			%		%		%		%
B	I	1	92.4	4.3	4.6	8.5	7.3	7.9	11.6	12.5	85.1	92.1	80.8	87.5
		2	89.7	4.8	5.3	8.5	7.0	7.8	11.8	13.1	82.7	92.2	77.9	86.9
		3	93.0	4.3	4.6	8.1	6.7	7.2	11.0	11.8	86.3	92.8	82.0	88.2
		4	90.4	4.1	4.5	7.5	5.1	5.6	9.2	10.1	85.2	94.4	81.2	89.9
		5	87.9	5.0	5.7	8.6	5.9	6.7	10.9	12.4	82.0	93.3	77.0	87.6
	Mean	1–3	91.7	4.4	4.8	8.4	7.0	7.6	11.4	12.4	84.7	92.4	80.3	87.6
	Mean	All	90.7	4.5	5.0	8.2	6.4	7.0	10.9	12.0	84.3	93.0	79.8	88.0
D	I	1	91.3	4.0	4.4	7.4	6.0	6.6	10.0	11.0	85.3	93.4	81.3	89.0
		2	88.7	4.2	4.7	7.1	5.5	6.2	9.7	10.9	83.2	93.8	79.0	89.1
		3	92.5	4.3	4.6	7.5	5.8	6.3	10.1	10.9	86.7	93.7	82.4	89.1
		4	89.6	4.2	4.7	7.4	5.1	5.7	9.3	10.4	84.5	94.3	80.3	89.6
		5	87.2	4.1	4.7	7.1	5.2	6.0	9.3	10.7	82.0	94.0	77.9	89.3
	Mean	1–3	90.8	4.2	4.6	7.3	5.7	6.3	9.9	10.9	85.1	93.7	80.9	89.1
	Mean	All	89.9	4.2	4.7	7.3	5.5	6.1	9.7	10.8	84.4	93.9	80.2	89.2
V	II	1	87.5	3.6	4.1	7.1	3.7	4.2	7.3	8.3	83.8	95.8	80.2	91.7
		2	88.3	3.6	4.1	6.6	4.1	4.6	7.7	8.7	84.2	95.4	80.6	91.3
		3	87.1	3.7	4.2	6.8	4.0	4.6	7.7	8.8	83.1	95.4	79.4	91.2
		4	90.6	3.8	4.2	6.9	4.6	5.1	8.4	9.3	86.0	94.9	82.2	90.7
		5	86.3	3.5	4.0	6.6	4.1	4.8	7.6	8.8	82.2	95.2	78.7	91.2
		6	88.6	3.5	4.0	6.6	3.9	4.4	7.4	8.4	84.7	95.6	81.2	91.6
		7	85.6	3.7	4.4	6.7	3.8	4.4	7.5	8.8	81.8	95.6	78.1	91.2
		8	89.4	3.6	4.0	6.7	3.6	4.0	7.2	8.0	85.8	96.0	82.2	92.0
	Mean	1–3	87.6	3.7	4.2	6.8	3.9	4.5	7.6	8.7	83.7	95.5	80.0	91.3
	Mean	4–8	88.1	3.6	4.1	6.7	4.0	4.5	7.6	8.6	84.1	95.5	80.5	91.4
	Mean	All	87.9	3.6	4.1	6.8	4.0	4.5	7.6	8.6	83.9	95.5	80.3	91.4
C	II	1	90.5	3.6	4.0	7.1	4.8	5.3	8.4	9.3	85.7	94.7	82.1	90.7
		2	91.1	4.0	4.4	7.2	3.7	4.1	7.7	8.5	87.4	95.9	83.4	91.5
		3	89.4	3.7	4.1	6.7	4.6	5.2	8.3	9.3	84.8	94.8	81.1	90.7
		4	92.7	3.7	4.0	6.8	5.0	5.4	8.7	9.4	87.7	94.6	84.0	90.6
		5	88.5	3.8	4.3	7.1	4.7	5.3	8.5	9.6	83.8	94.7	80.0	90.4
		6	91.3	3.8	4.2	7.1	4.6	5.0	8.4	9.2	86.7	95.0	82.9	90.8
		7	87.4	3.7	4.2	6.8	4.2	4.8	7.9	9.0	83.2	95.2	79.5	91.0
		8	91.5	3.7	4.0	6.6	4.1	4.5	7.8	8.5	87.4	95.5	83.7	91.5
	Mean	1–3	90.3	3.7	4.1	7.0	4.4	4.9	8.1	9.0	85.9	95.1	82.2	91.0
	Mean	4–8	90.3	3.8	4.2	6.9	4.5	5.0	8.3	9.2	85.8	95.0	82.0	90.8
	Mean	All	90.3	3.7	4.1	6.9	4.5	5.0	8.2	9.1	85.8	95.0	82.1	90.9
J	II	1	85.1	3.2	3.8	6.7	4.6	5.4	7.8	9.2	80.5	94.6	77.3	90.8
		2	85.7	3.7	4.3	7.0	4.4	5.1	8.1	9.4	81.3	94.9	77.6	90.6
		3	83.6	3.3	3.9	6.6	4.6	5.5	7.9	9.4	79.0	94.5	75.7	90.6
		4	86.5	3.9	4.5	7.9	4.3	5.0	8.2	9.5	82.2	95.0	78.3	90.5
		5	82.8	3.5	4.2	7.1	4.6	5.6	8.1	9.8	78.2	94.4	74.7	90.2
		6	85.4	3.4	4.0	6.9	4.6	5.4	8.0	9.4	80.8	94.6	77.4	90.6
		7	82.0	3.4	4.2	6.8	4.6	5.6	8.0	9.8	77.4	94.4	74.0	90.2
		8	85.6	3.2	3.7	6.5	4.6	5.4	7.8	9.1	81.0	94.6	77.8	90.9
	Mean	1–3	84.8	3.4	4.0	6.8	4.5	5.3	7.9	9.3	80.3	94.7	76.9	90.7
	Mean	4–8	84.5	3.5	4.1	7.0	4.6	5.4	8.1	9.5	79.9	94.6	76.4	90.5
	Mean	All	84.6	3.4	4.1	6.9	4.6	5.4	8.0	9.5	80.0	94.6	76.6	90.5

experiment, who had, therefore, more available calories. There may have been many reasons for the differences in the results of the two experiments, but the slight variations in the composition of the diets was probably the main reason. Therefore, the results of the two experiments could not be averaged, since erroneous conclusions might result.

In attempting to find the effect of diet on calorie balance, it seemed wise to base the discussion entirely on the results found in the second experiment, because there were fewer variables in the diets used here. The average calorie values in both diets were identical and the constituents of the diets were also the same, except for slight changes in the second one, made to increase the protein content and to keep the calories at the same level (Hawks, Dye and Bray, '37)[4]

To determine whether or not the period-to-period fluctuations in diet calories influenced the number of calories retained, averages of the intake and retention values per kilogram for all of the children, for each separate period, were calculated. This was possible because the children received exactly the same foods and the same number of calories per kilogram. A comparison of these averages (fig. 1)[5] showed that the small diet fluctuations were reflected in the retention values and that even the change to the high protein diet did not influence this relationship. The constant percentage of the intake calories retained during the several periods on each diet (tables 1 and 2), as well as the high correlations between intake and retention (0.936 on the 3-gm. and 0.940 on the 4-gm. protein diets), also substantiated this relationship. Therefore, the number of available calories would seem to be dependent upon the intake calories.

Nevertheless, the change to the high protein diet also affected the calorie retention. The percentages of the intake retained were lower then those on the medium protein diet

[4] The only differences in the 4-gm. protein diet were the addition of egg white and gelatin and the omission of a portion of the butter.
[5] Although the discussion includes only the results of experiment 2, the figure gives the data for both experiments.

(table 2) because the high protein diet increased the total number of calories eliminated, both in urine and feces. These increases were not large, but the urine values were 1% and the feces values from 0.0 to 0.3% higher. It is probable that the increased nitrogen eliminated was responsible for almost
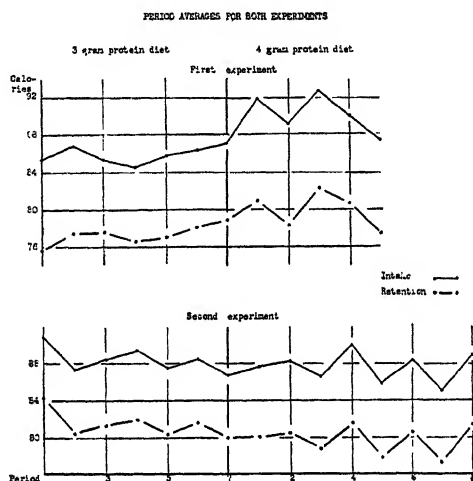

Fig. 1 The relationship between the intake and retention values obtained by averaging the per kilogram data for all of the children for each separate period.

TABLE 3

Increase in calorie values per kilogram on high protein diet as analyzed and as calculated from nitrogenous end products

CHILD	URINE CALORIES		FECES CALORIES	
	Analyzed	Calculated	Analyzed	Calculated
V	0.9	0.915	0.3	0.250
C	0.9	0.896	0.1	0.125
J	0.8	0.777	0.0	0.075

all of the increased calorie values in the excreta. Several facts substantiate this. The first is, that the increases in the values for analyzed calories and for those calculated (table 3) from the nitrogenous end products (Hawks, Bray and Dye, '37) were practically the same. Second, the calories excreted in the urine seemed to depend upon the nitrogen in the diet, since the correlation between these values was 0.932. Third,

the average ratios between urinary calories and nitrogen were practically the same for each child on the two diets (tables 1 and 2). Fourth, the correlations between the values for fecal calories and the dry weight of the feces, which the increase in nitrogenous end products probably caused, were 0.917 on both diets.[6] Therefore, all of the above facts seem to indicate that nitrogen may have been the sole cause for the increased excretory calories on the high protein diet.

The increased proportion of the calories excreted on the high protein diet indicated that a definite proportion of calories was not always eliminated in the excreta. In the present experiment, the average percentage excreted in urine and feces varied from 7.3 to 12.0. This difference of nearly 5% shows the necessity for actual analyses of the excretory material for most accurate results, since the practice of either subtracting 10% from the intake values, or of using physiological values, may introduce considerable error.

The higher proportional output of calories on the high protein diet caused a decrease in the number of calories retained, or a decrease in the number available for basal metabolic needs, growth and activity. Nevertheless, the children gained weight at a more rapid rate during this period than they had on the medium protein diet, when they had had a larger number of available calories (table 4). Although this seemed impossible, the results conformed with those which Forbes et al. ('35, '38 and '39) obtained for rats in a similar experiment. They fed equicaloric diets which contained protein in amounts varying from 10 to 45%. As the protein content of the diets increased, the metabolizable energy, or total calories retained, decreased. Even with the lowered available energy, the rate of weight gains was progressively greater, until the diet contained 25% protein. Above that level, the weight gain of the

[6] If the increased nitrogen in the feces caused the entire calorie increase, other constituents of the diet besides nitrogen must have been eliminated in relatively constant amounts. This fact adds some evidence to the contention, discussed in connection with the nitrogen data (Hawks, Bray and Dye, '38), that there is a constant fraction eliminated in the feces.

rats diminished. The diets of the children in the present experiment contained comparable percentages of protein, approximately 13 and 18% for the 3- and 4-gm. protein diets respectively. Therefore, under similar conditions, both rats and children gained at an increased rate with increase in the protein content of the diet.

It is impossible to tell from the present experiment whether the reduction in the available calories gave fewer calories for growth, produced a lower basal metabolism, or curtailed activity. Forbes and his associates suggested that the activity of their rats might have been reduced, because calculated heat production, which represents basal metabolism plus activity, became lower as the protein in the diet increased and at the same time basal metabolism values remained constant. Since several investigators (Wang and her associates, '30; DuBois,

TABLE 4

Average gain in weight per day

DIET	B	D	V	C	J
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Medium protein	1.43	0.95	3.81	4.29	2.38
High protein	5.33	4.67	4.58	8.75	8.75

'36), have reported that the protein content of the human diet does not influence the basal metabolism, it may be that these same facts are true for the children in the present study. As far as could be determined, however, the activity of the children remained the same on the two diets, since they spent approximately the same time each day in active exercise and followed a regular routine.

There may be several reasons for the increase in the weight gains of the children on the high protein diet. Since the diet increased the coefficient of digestibility of nitrogen and the fraction of the intake absorbed was raised as much as 2.6% (Hawks, Bray and Dye, '38), it may be possible that the egg white and gelatin used to increase the nitrogen content of the diet were more effective for producing weight gains than calories from other sources, even if the actual number of

calories absorbed was lower. The storage of a larger number of grams of protein may also have increased the water content of the body and thus increased the weight gains. These facts may indicate that 3 gm. of protein per kilogram of body weight may not be sufficient for optimum growth as well as for optimum protein storage. Judging from the work of Forbes et al., even a 4-gm. protein diet may not contain enough protein to produce the most efficient utilization. More work on these subjects, however, would have to be done before definite conclusions could be drawn.

Although the higher protein content of the diet did produce changes in calorie balances, it seemed to cause no differences in the variability of the calorie data. The urine and feces values remained strikingly constant from period to period, as they had on the medium protein diet. The absorption and retention values fluctuated to the same degree and in the same manner, as indicated by the high correlations with intake, 0.939 and 0.940, for absorption and retention respectively.

Thus it may be said that both calories and protein influenced calorie utilization. The availability seemed to be directly proportional to calorie fluctuations in diet, irrespective of the change in the protein content, but at the same time the increase in protein reduced the percentage of the intake calories absorbed and retained.

SUMMARY

1. Five pre-school children served as subjects for two long-time balance experiments, in each of which they received two diets, the first containing 3 and the second 4 gm. of protein per kilogram of body weight.

2. The increase in the protein content of the diets did not change the constant level of calorie utilization. Therefore, preliminary periods before the high protein diets seemed unnecessary in these experiments.

3. The children in each experiment reacted in the same manner, although there were slight differences in the results of the two experiments.

4. The period-to-period variations for excretory values remained exceedingly constant on both diets and the absorption and retention figures varied in the same manner and to the same degree as the intake values.

5. The change from the 3- to the 4-gm. protein diet affected calorie balance as follows: (a) It increased the nitrogen content of the excreta, thus increasing the actual number of calories eliminated. (b) It increased the average proportion of the intake calories eliminated from 7.3 to 12.0%. Thus, subtracting 10% from the intake values to care for excretory losses does not always give accurate results. (c) It reduced the actual number, as well as the percentage, of the intake calories available for body needs, but at the same time it produced greater weight gains in the children.

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MINIMUM VITAMIN A AND CAROTENE REQUIREMENTS OF MAMMALIAN SPECIES [1]

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ONE FIGURE

This paper presents data on the vitamin A and carotene requirements of the horse, re-evaluates and summarizes previous data from this laboratory and correlates all this material with other information pertinent to the requirements of mammalia in general.

EXPERIMENTAL PART

The methods of procedure were similar to those used in the experiments with cattle (Guilbert and Hart, '35) and cattle, sheep, and swine (Guilbert, Miller and Hughes, '37).

Rations

The basal rations used for the nine horses upon which we have quantitative data are shown in table 1. Barley straw was used for roughage during the depletion period for horses nos. 1, 2, 3, and 4. During the later part of the experimental period for horse no. 3, straw was omitted from the ration. No carotene analyses were made on the concentrate mixtures or straw during the period when the animals' vitamin-A stores were being depleted. During the experimental periods when carotene or vitamin-A requirements were being determined, com-

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[1] This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Bureau of Animal Industry, July 1, 1929.

posite samples were analyzed and total carotene intake was computed on the basis of both the supplement and the basal ration. It was found that the animals thrived without the addition of roughage, and to lower the carotene content of the ration, straw was omitted in later experiments. All of the animals chewed on fences and mangers, which necessitated their protection with wire and metal strips. Omission of roughage may have increased this activity. Except for the substitution of straw for hay, the rations of the first four animals were typical of widely-used horse rations.

TABLE 1

Basal rations

| | HORSE NUMBER | | | | |
FEEDS	1 and 2	3 and 4	5	7 and 8	10 and 12
Dried molasses beet pulp	..	..	..	55	53.5
Rolled barley	30	30	30	10	10
Rolled oats	30	30	30	10	10
Wheat bran	30	30	30	10	10
Linseed oil meal	10	8.5	5	10	10
Cottonseed meal	..	..	5	5	5
$CaCO_3$	..	1.5	..	..	1.5
Straw	ad lib.	ad lib.	..	..	..

Depletion time

The purebred Percherons used were raised on the University Farm. Previous to being placed on experiment, they had had normal rations including abundant alfalfa hay and pasture.

The age of animals nos. 1 to 4 inclusive when started on experiment varied from 284 to 376 days and the time elapsing before the onset of night blindness varied from 492 to 627 days. The depletion time for nos. 5 to 12 inclusive was significantly less than for the first four animals, presumably because their basal rations without straw contained less carotene. The age of these animals when placed on experiment varied from 119 to 444 days and the depletion time varied from 265 to 439 days.

Symptoms

The first detectable symptom in the horse, similar to that of other species, was night blindness. The horse normally appears to have particularly good night vision and has an acute sense of direction and memory for the position of obstacles. This latter trait frequently made it difficult to ascertain whether or not animals could see when they were in quarters to which they were accustomed. Moreover, once they had run into obstacles they were cautious to avoid repeating their mistakes, thus making routine testing for night blindness more difficult than with cattle, sheep, and swine. A series of movable panels set up in an alley-way adjoining the pens formed a varying maze that permitted us to judge more critically visual acuity. Tests generally were made when black 144 point Ebar light condensed capital letters on white background were discernible at 3 to 6 feet.

When completely night blind, the animals carried their heads high, with nose extended, or close to the ground, feeling their way around. They moved rather freely, however, in their own lots. If the gate into the alley was opened, the horses were driven through only with difficulty. Time after time they could be driven at a trot up to the normal position of the gate, at which point they stopped abruptly, whirled, always just missing the normal position of the gate. This strikingly demonstrated their uncanny memory for position of obstacles as well as their complete inability to see in light that plainly revealed surrounding objects to normal persons and horses. Once they were placed outside their lot and forced by driving to move rapidly, they ran head on into panels placed in their path. They often became completely lost and greatly excited. Partially defective vision could be distinguished by the distance at which they were able to see obstacles and alter their course to avoid collision.

If vitamin-A therapy were not instituted after the appearance of night blindness, symptoms progressed gradually over a period of 3 to 4 months. The hair became rough, and later

there was clouding of the corneas and impaired daylight vision. Excessive lachrymation rather than xerophthalmia was exhibited. At this stage the eyes of nos. 2 and 3 were examined by Dr. D. K. Mills, ophthalmologist of the Woodland Clinic. The change in the corneas was found to be primarily in the deeper layers rather than on the surface. This keratinization appeared first as streaks and later appeared to involve equally the whole cornea. Comparison of the eyes at this stage with those of normal animals revealed no swelling nor elevation of the disc. The horses observed did not show the susceptibility to convulsions that is exhibited by cattle. On one occasion, however, no. 2 staggered and went down just prior to the onset of estrus. Vitamin-A deficient horses did show some nervousness and increased irritability when touched. No. 3, a stallion, bred filly no. 2 several times when both were showing night blindness. This confirms other observations that early stages of deficiency do not affect estrus or libido. We have no record of impregnation, however, under these conditions.

Horse no. 3 at about the onset of corneal involvement developed a nasal discharge and snoring, which became progressively worse as the deficiency advanced. The appetite declined and the animal, after losing over 100 pounds weight, became weak. Ten days after giving small doses (5 cc. daily of cod liver oil containing 2600 I.U. per gram), the corneas were clear and the eyes stopped watering; breathing became easier; appetite and general condition rapidly improved. Night vision became normal within 30 days. The snoring never entirely disappeared. After cod liver oil feeding was stopped, the same general sequence of symptoms reappeared. Snoring became worse and breathing difficult. Six weeks after cessation of vitamin A therapy breathing became very difficult. The animal suddenly began bleeding and discharging from the nostrils, went down, and did not get up for several hours. Later he got up without help and breathing was easier. Diarrhea developed during the next week, appetite declined, the animal became weaker and a month later developed acute difficulty in breath-

ing and died. Post mortem examination revealed no obstruction in the nasal passages.

Animal no. 2 gradually became weak, and finally, unable to rise, was killed in extremis. Animal no. 4 developed night blindness during the last month of gestation, had difficulty in labor, and died following removal of a dead foal that was abnormally presented. Horse no. 1 became so lame from bone and joint abnormalities that he could not get up and was killed. This animal was receiving cod liver oil prior to autopsy and had some vitamin-A storage. The other animals gave negative tests for vitamin A in the liver. Animal nos. 5 to 12 inclusive were still living at the time of writing.

MINIMUM VITAMIN A AND CAROTENE REQUIREMENTS

Dehydrated alfalfa meal was used as a source of carotene. The methods of sampling, feeding, and analysis were the same as reported by Guilbert, Miller and Hughes ('37). The cod liver oil used was also from the same batch used in their experiments. Goss and Guilbert ('39) showed that no change had occurred in the extinction coefficient of this oil which had been stored at $-10°C$. They re-evaluated the spectrophotometric analysis on the basis of the extinction coefficient $E_{1\,cm}^{1\%}$ 328 mμ = 2100 found by Holmes and Corbet ('37) for crystalline vitamin A. Parallel rat experiments with this oil and U.S.P. Reference cod liver oil showed that it contained 2600 I.U. per gram, an amount slightly less than that indicated by comparative spectrophotometric analyses of the two oils.

When quantitative dosage was begun after the onset of night blindness carotene analyses were run at frequent intervals on composite samples of both straw and concentrate mixtures. The straw contained more carotene than that used in previous experiments and varied from 0.05 to 0.10 mg. %. The concentrate mixtures consistently contained between 0.01 and 0.02 mg. % of pigment measured colorimetrically as carotene. These amounts in the basal rations including straw, contributed between 5 and 10 micrograms of carotene per kilogram body weight as shown in table 2.

TABLE 2

Minimum carotene and vitamin-A requirements of the horse
(source of carotene, alfalfa meal; source of vitamin A, cod liver oil)

ANIMAL NUMBER	DURATION OF PERIOD	INTAKE	RESULTS
	days	*μg. per kg.*	
	..	Carotene 5 to 10 [1]	Became night blind
1	14	Carotene 29 to 33	Vision became normal
	60	Carotene 19 to 26	Vision borderline to normal
	..	Carotene 5 to 10 [1]	Became night blind
	22	Carotene 30 to 35	Vision became normal
2	60	Carotene 20 to 26	Vision remained normal
	41	Carotene 5 to 10 [1]	Became night blind
	27	Carotene 18 to 24	Partially night blind
	..	Carotene 5 to 10 [1]	Became night blind
3	27	Carotene 15 to 23	Partially night blind
	20	Carotene 25 to 30	Vision became normal
	27	Carotene 5 to 10 [1]	Became night blind
	..	Carotene 5 to 10 [1]	Became night blind
4	32	Carotene 18 to 23	Vision became normal
	42	Carotene 5 to 10 [1]	Became night blind
	41	Vitamin A 1.5 [1]	Became night blind
2	59	Vitamin A 5.1	Vision became normal
	41	Vitamin A 1.5 [1]	Became night blind
	27	Vitamin A 1.5 [1]	Became night blind
	41	Vitamin A 7.3	Vision became normal in 27 days
	145	Vitamin A 1.5 [1]	Became night blind in 73 days
3	124	Vitamin A 0.4 [1]	Advanced symptoms developed
	72	Vitamin A 5.3–5.8	Marked improvement, sight normal
	30	Vitamin A 0.4 [1]	Became night blind
5	31	Vitamin A 4.2	Vision became normal
	11	Vitamin A 0.4 [1]	Became night blind
7	15	Vitamin A 4.5	Vision became normal
	32	Vitamin A 3.8	Vision became normal
	55	Vitamin A 3.3	Became partially night blind
	30	Vitamin A 3.7	Vision remained partially defective
8	10	Vitamin A 4.1	Vision remained slightly defective
	9	Vitamin A 0.4 [1]	Became completely night blind
	21	Vitamin A 4.3	Remained partially night blind
	32	Vitamin A 5.3	Vision became normal
	8	Vitamin A 3.7	Vision became normal
	59	Vitamin A 3.0	Borderline to normal vision
10	70	Vitamin A 0.4 [1]	Slowly became night blind
	11	Vitamin A 4.4	Vision became normal
	11	Vitamin A 0.4 [1]	Became night blind
12	17	Vitamin A 4.3	Vision became normal

[1] Furnished by basal ration and straw.

In the experiments on vitamin-A requirement, the basal ration with straw was used only with animal no. 2 and the first trial with no. 3. In all other tests, straw was omitted. Since previous experiments have shown that the amount of carotene to meet minimum requirements is approximately five times that of vitamin A, the vitamin-A equivalent of the carotene furnished by the basal ration was computed on this basis. The average food consumption was about the same with respect to the body weight for the various animals. The average vitamin-A equivalent of the carotene in the basal ration with straw and without straw was roughly 1.5 and 0.4 microgram per kilogram body weight respectively as shown in table 2. The figure for vitamin-A intake during each test period includes the calculated vitamin-A equivalent of the carotene in the basal ration.

The adequate and subminimum carotene levels shown in table 2 overlap somewhat, largely because the extreme range of carotene contained in the basal ration was used in reporting the data. It is evident, however, that the minimum level lies between 20 and 30 micrograms per kilogram body weight which corresponds closely with previous results on other species.

In some instances individuals progressed very slowly from the first evidence of impaired vision to complete night blindness. The first trials with cod liver oil on nos. 8 and 10 may have been started while vestiges of reserves were being slowly withdrawn from fat or liver, as the dosage required to restore normal vision was less than that found with the same animals in later trials. With these exceptions, table 2 shows that vitamin-A levels less than 4 micrograms per kilogram body weight were borderline and that the adequate level lay between 4.2 and 5.3 micrograms per kilogram body weight.

It is significant that the higher level supplied by 5 cc. of cod liver oil containing 0.063% vitamin A as measured by the Hilger vitameter or 2600 I.U. per gram as compared with U.S.P. reference cod liver oil restored animal no. 3 to thrifty condition after he had progressed to a critical condition on

the basal ration. The weight of this animal varied between 1060 and 1165 pounds during the period.

DISCUSSION

The same batch of cod liver oil was used in all of our experiments. It was kept at low temperature and analyses run from time to time revealed no change in its value. For the cattle, sheep, and swine experiments, it was analyzed by means of a Hilger Vitameter using the value $E_{1\,cm}^{1\%}$ 328 mμ = 1600. In order to compare these data with those found for horses and rats they have been re-evaluated on the basis of $E_{1\,cm}^{1\%}$ 328

TABLE 3

Summary of data from this laboratory on minimum vitamin-A and carotene requirements of various species

SPECIES	DAILY INTAKE PER KILOGRAM BODY WEIGHT			
	Vitamin A		Carotene	
	$\mu g.$	*I.U.*	$\mu g.$	*I.U.*
Cattle	5.1–6.4	21–27	26–33	43–55
Sheep	4.3–6.3	17–26	25–35	42–58
Swine	4.4–5.7	18–24	25–39	42–65
Horse	4.2–5.3	17–22	20–30	33–50
Rat	4.6–5.3 [1]	....	15–20	25–33
Rat	3.8–4.6 [2]	18–22	....	....

[1] Data based on cod liver oil that was also used in cattle, sheep, swine and horse experiments.

[2] Data based on U.S.P. Reference cod liver oil.

mμ = 2100 reported for crystalline vitamin A (Holmes and Corbet, '37). The rat experiments of Goss and Guilbert ('39) compared the biological activity of this cod liver oil with that of U.S.P. Reference cod liver oil. This enables us also to express the requirement for vitamin A in international units. The data expressed both by weight and in international units are summarized in table 3.

The minimum requirement for cattle, sheep, swine, and horses was defined as the lowest level per unit of body weight that prevented any detectable degree of nyctalopia under light conditions as described in this paper. The minimal level for

the rat was that which just sufficed to prevent any abnormal degree of cornification of the vaginal smears. In all cases, these minimum levels provided for normal growth and general well-being but permitted little or no storage. It is significant that with the same cod liver oil as the source of vitamin-A, the data for the rat and for the other species agree though different criteria of sufficiency were used. Since no significant storage occurred in any case, it is evident that the onset of night blindness and of vaginal cornification occur at the same level of deficiency.

The rat experiments showed that the U.S.P. Reference oil possessed slightly greater vitamin-A potency although both oils happened to have equal values as measured by the vitameter. We assume from this that the Reference oil contained less irrelevant material that contributed to light absorption at the vitamin-A wave length.

The minimum carotene level for the rat was slightly less than that found for other species, possibly due to more efficient absorption from the intestinal tract. These levels for the rat, however, varied from sub-minimum to adequate while all levels of dosage given for other species prevented night blindness. The lowest level in these cases may be taken as most nearly representing the actual minimum.

These data are in such good agreement that it is desirable to explore the possibility of similar consistent relationships concerning requirement for other functions such as significant storage, reproduction and optimal dark adaptation.

Previous data from this laboratory have shown that the minimum levels as defined above are not adequate for reproduction. Cows were able to produce live young at term but the calves were weak and soon died. Three to four times the minimum vitamin-A level beginning with the last month of pregnancy resulted in normal calves and the mothers supplied sufficient vitamin in the milk for normal growth of the calves for at least 3 months. Three to four times the minimum carotene level for the mother also resulted in birth of normal

calves. Deficiency symptoms, however, developed in the calves within 3 to 4 weeks although the cows remained normal.

Over extended periods of time levels of intake of about three times minimum vitamin-A intake resulted in storage. Five times the minimum carotene were required before significant storage could be demonstrated in rats after a 90-day dosage period. The evidence, therefore, indicates that about three times the minimum vitamin-A level and five times the minimum carotene level is about minimum for significant storage and reproduction and that with the aid of reserves established during non-lactating intervals should provide enough vitamin A in the milk to support young during the nursing period.

It is evident that the ratio of the relative efficiencies of vitamin A and of carotene widens with increasing levels of intake. On the basis of our analysis of U.S.P. Reference oil and its assigned biological value, 0.21 microgram approximates 1 I.U. At the biological unit level therefore the ratio of efficiency of vitamin A to carotene by weight is about $3:1$; at the level to meet our definition of minimum about $6:1$ and at the minimum level that results in significant storage and successful reproduction about $10:1$. So far as mammals are concerned it is obvious that an international unit of carotene and an international unit of vitamin A have equality only under the conditions of the biological test. Double standards for requirements must be recognized, one for carotene and one for vitamin A, and both must be considered in evaluating the status of a dietary furnishing both sources.

The following human data that were reported in terms of intake per unit of body weight or suitable for calculating on this basis are briefly summarized for further comparison of general relationships. Aykroyd and Krishman ('36) reported eye symptoms in 27% of a group of 436 children who were receiving an estimated intake of 20–30 micrograms carotene per kilogram body weight. Edmund and Clemmesen ('37) found poor dark adaptation and some dysadaptation in men receiving about 18–20 I.U. per kilogram body weight. Jeghers ('37) likewise reported subjective night blindness in subjects

who for a long time had ingested daily an estimated 15–18 I.U. per kilogram body weight. Although these levels in some cases cannot be closely evaluated because both vitamin A and carotene contributed to the total, they appear to coincide with levels found by us to be borderline or deficient. Levels of intake three to five times our minimum and which suffice for

TABLE 4

Summary of vitamin A and carotene requirements in relation to body weight and an illustration of requirements of a 70 kg. individual

REQUIREMENT	VITAMIN A PER KILOGRAM BODY WEIGHT		CAROTENE PER KILOGRAM BODY WEIGHT		TOTAL FOR 70 KG. INDIVIDUAL			
					Vitamin A		Carotene	
	μg.	I.U.	μg.	I.U.	μg.	I.U.	μg.	I.U.
Minimum for normal growth, freedom from clinical symptoms, little or no storage	4	20	25	40	280	1400	1750	2800
Minimum for significant storage, optimal dark adaptation, and reproduction	12	60	125	200	840	4200	8750	14,000
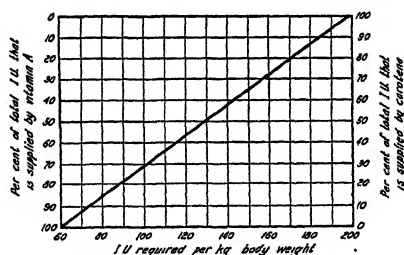
Fig. 1 Number of international units daily per kilogram body weight to satisfy minimum requirements for significant storage, optimal dark adaptation and reproduction when supplied by varying proportions of vitamin A and of carotene.

storage and reproduction have also been reported to be adequate for general well-being or for optimal dark adaptation in human subjects (Jeans, Blanchard and Zentmire, '37; Jeghers, '37; Lewis and Barenberg, '38; Booher, Callison and Hewston, '39). Crimm and Short ('37) and Phillips and Bohstedt ('38) presented data on dogs and rabbits respectively that fall within the range of intakes under discussion.

Because of the agreement of all these data, we propose and present in table 3 general daily requirements for vitamin A and for carotene and from these illustrate in table 4 that for a 70 kg. individual. The basic vitamin-A figures are given in round numbers converted to the basis of U.S.P. Reference oil. Figure 1 is presented to show the total requirement in international units to meet the minimum for significant storage, optimal dark adaptation and reproduction when supplied by varying proportions of vitamin A and of carotene.

SUMMARY

Data are presented on time required to deplete vitamin-A reserves of horses and on their minimum requirement for vitamin A and for carotene. The more conspicuous symptoms of vitamin-A deficiency in the horse are described.

The data previously published on vitamin-A requirement of cattle, sheep, and swine were re-evaluated and a summary table presents all of the data from this laboratory on vitamin-A and carotene requirements of various species expressed both by weight and in international units.

The agreement of these and other data, with regard to the minimum to prevent earliest symptoms and also the higher levels necessary for storage and reproduction in animals and optimal dark adaptation in man are discussed. The necessity for two standards for expressing requirements, one for vitamin A and one for carotene is pointed out. These are illustrated by means of a table and a graph.

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CHRONIC SELENIUM POISONING OF RATS
AS INFLUENCED BY DIETARY PROTEIN

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TWO FIGURES

In the course of preliminary experiments concerned with the nutritional aspects of selenium poisoning in rats, Lewis and Gortner ('37) observed that those animals receiving casein at a level of 30% in the diet were much less susceptible to the effects of added sodium selenite than were others receiving only 6% of dietary casein. This protective action of the high protein diet was manifested in the growth, longevity, and general appearance of the animals. About the same time Moxon ('37) reported that high protein diets afforded more protection against selenium than did diets low in protein. When rats were fed diets containing 10, 20, and 55% of casein together with 37.5 p.p.m. of selenium as sodium selenite, those receiving the most protein grew more than twice as fast as others on the low protein diet. Recently Smith ('39), in a detailed study of the influence of dietary constituents on chronic selenium intoxication in rats, observed again the protective action of high levels of protein. Selenium was present in these diets as the form occurring naturally in seleniferous flour.

The present experiments were undertaken in order to determine whether the earlier observed protection against ingested selenium by high levels of casein could be obtained with other proteins. Accordingly, diets containing such proteins as lactalbumin, gelatin, and edestin were studied in comparison with one high in casein and another low in protein content.

105

EXPERIMENTAL

The compositions of the experimental diets are shown in table 1. The low protein diet used was the sulfur-deficient ration of White ('36) to which cystine was added in amounts sufficient to increase the sulfur content so as to be equal to that of the 30% casein diet. The various high protein diets were all derived from this basal diet; sufficient other protein was added to the 6% of casein already present to make a

TABLE 1

*Composition of experimental diets**

The values indicate parts by weight and are approximate percentage units. In addition to these diets each rat received 3–4 drops of cod liver oil and one tablet (390 mg.) of pressed brewers' yeast daily.

DIET	LP_{se}	C_{se}	G_{se}	E_{se}	L_{se}
Casein	6	30	6	6	6
Gelatin	0	0	24	0	0
Edestin	0	0	0	24	0
Lactalbumin	0	0	0	0	24
O–M salt mixture cont'g 875 p.p.m. Se	4	4	4	4	4
Sucrose	15	15	15	15	15
Cornstarch	50	26	26	26	26
Lard	25	25	25	25	25
Cystine	0.68	0	0.68	0	0

* The proteins used were the following commercial products:
   Casein—Labco vitamin-free casein
   Gelatin—Eimer and Amend, U.S.P. granular
   Edestin—Eimer and Amend, pure
   Lactalbumin—Labco lactalbumin, No. 7HA

final concentration of 30% protein, this being substituted for an equivalent weight of starch. Selenium, as c.p. sodium selenite, was incorporated in the Osborne-Mendel salt mixture in such a concentration as to give a selenium level of 35 p.p.m. in all of the selenium-containing diets when fed. The selenium-free control diets were identical with those in the above table except that no selenium was incorporated in the salt-mixture. In figure 1 they are designated by the same letters as the seleniferous diets except that the subscript 'se' is omitted.

Young albino rats of inbred stock obtained from the Germantown, Pennsylvania colony were used throughout these experiments. At the age of 25 to 30 days they were transferred to individual raised-bottom wire cages and fed diet C for 4 to 7 days in order to accustom them to an artificial diet. When they were changed to control or seleniferous diets their weights ranged from 50 to 70 gm. The daily food intakes were recorded, and each rat was weighed twice weekly. The experiments were continued for periods ranging from 6 to 8 weeks,
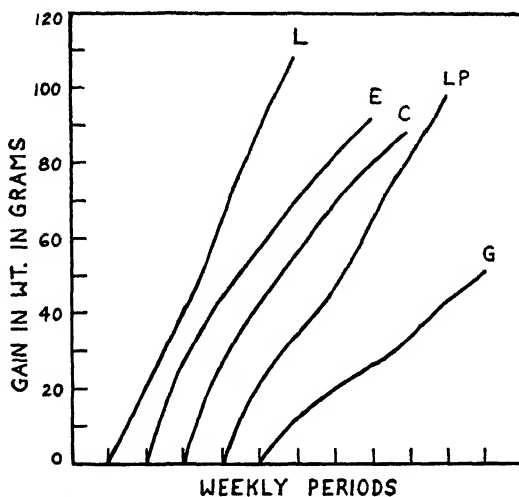
Fig. 1   Average growth curves for male control rats.

Each curve represents 2 or 3 animals. The letters with each curve designate the diet received.

The results of feeding the selenium-free control diets are shown in figure 1. It is evident that good growth is obtained with the low-protein diet as well as with those high in casein, lactalbumin and edestin. The rats receiving the high-gelatin diet, however, grew only about one-half as rapidly as the others, and their fur tended to become yellow and greasy in contrast to the animals on the other diets.

Figure 2 depicts the average growth curves of male and female rats on the 35 p.p.m. selenium diets. The same general

trends were observed for the two sexes with the exception that growth was not as pronounced in the females. Although only from 7 to 14 rats were placed on each of these diets, numbers admittedly not large, it is felt that the data are of such a consistent nature as to merit their being reported at this time. It should be emphasized that the diets high in lactalbumin, gelatin, and edestin all contained a basal level of
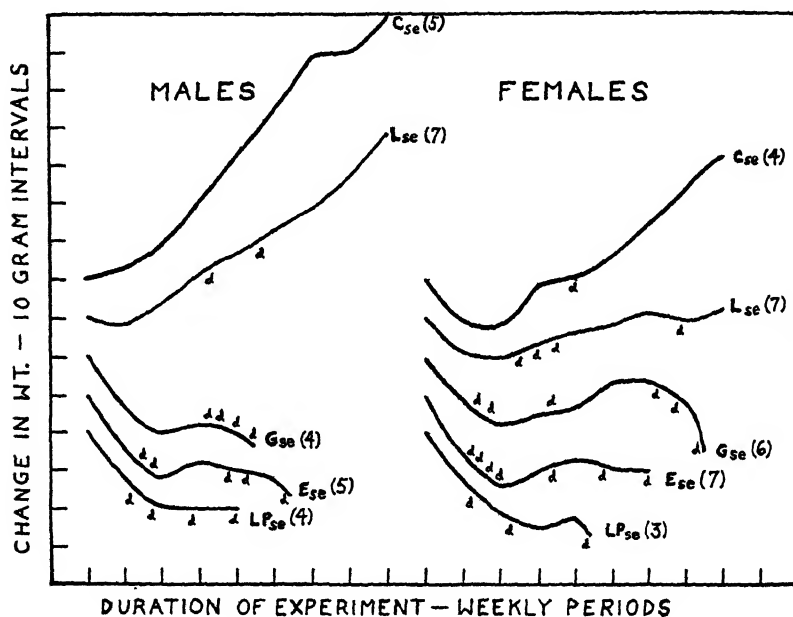
Fig. 2 Average growth curves for rats on seleniferous diets.

Numbers in parentheses represent number of animals on the diet. A small "d" indicates the death of an animal. The letters with each curve designate the diet received.

6% casein, so that the effects of these proteins on growth must be considered as supplementary effects.

It is seen that casein facilitated fair growth, and at the end of the 8-week experimental period all of the males and all but one of the females appeared healthy and were continuing to gain in weight. Examination of the internal organs at the end of this time showed gross pathological changes to a greater or

lesser extent in about 60% of the animals, the females being more severely affected than the males. The observed pathological changes included shrivelled, mottled, and granular appearance of the liver, hypertrophy of the spleen, and hypertrophy of the heart. Histological examinations of the livers showed some congestion. The testes were large and firm, and histological observations showed apparently normal spermatogenesis.

Lactalbumin also appeared to offset selenium intoxication to some extent, but the results were not so consistent as those obtained with casein. Of the seven males on the lactalbumin diet, four grew at a rate comparable to the rats receiving the high level of casein, while the others did poorly, two succumbing within 5 weeks. With the females, three showed fair growth during the experimental period while the remaining four expired before the end of this period. Accordingly, eight of the fourteen rats on this diet were alive and growing at the end of 8 weeks. The gross pathology of the internal organs was much the same for this group of rats as it was with those receiving the diet C_{se}.

With the other diets, however, the results were quite different. The edestin and gelatin diets failed to promote growth and offset the toxic manifestations of the selenium and were in nearly all respects comparable to the low-protein diet. In no instance did the survival period extend beyond 53 days, most of the deaths occurring between 9 and 32 days. Except for those animals which succumbed within 2 weeks after being placed on selenium-containing rations, gross pathology of the organs was more evident in these groups than in the groups receiving diets C_{se} and L_{se}. Atrophy and cirrhosis of the liver were observed in 75% of the animals, and a similar percentage showed varying amounts of edemal fluid or blood in the pleural cavity, peritoneal cavity, or intestine. The blood was invariably thin and watery. The testes were flabby and undeveloped. Other organs such as the heart and spleen were usually very small but occasionally were decidedly hypertrophied.

Since gelatin is known to be practically devoid of trypto-phane and sulfur, further experiments were carried out with fourteen rats in which 0.1% tryptophane and 0.84% methio-nine were included in a high-gelatin diet, the latter amino acid being substituted for the cystine in. diet G_{se}. However, the resulting failure of growth and early mortality were just as pronounced in this group as in the one that received the high gelatin plus cystine diet (G_{se}).

The average daily intake of food varied greatly according to the diet. The animals on the non-seleniferous control diets

TABLE 2

Average food and selenium intakes of male rats on control and on selenium-containing diets.

DIET	NUMBER OF RATS	AVERAGE DAILY FOOD INTAKE		AVERAGE DAILY INTAKE OF Se	
		Range	Average	Range	Average
		gm.	*gm.*	*mg.*	*mg.*
LP	2	7.3–9.5	8.4		
C	2	7.2–7.5	7.4		
L	2	6.2–7.6	6.9		
G	3	6.6–7.2	6.9		
E	2	7.1	7.1		
LP_{se}	4	1.6–2.5	2.1	0.056–0.088	0.074
C_{se}	5	3.8–5.8	4.7	0.133–0.203	0.164
L_{se}	7	2.0–5.8	3.9	0.070–0.203	0.136
G_{se}	4	2.1–3.0	2.6	0.074–0.105	0.091
E_{se}	5	1.3–2.5	2.1	0.046–0.088	0.074

ingested, on the average, 6.5 to 7.5 gm. of food per day. When selenium was present in the diets, smaller amounts were con-sumed, as shown in table 2. It is evident from these data that, as has been pointed out previously by Franke and Potter ('35) and Franke ('35), growth is directly proportional to the food intake despite the increased consumption of selenium accompanying the higher levels of food intake.

DISCUSSION

The data clearly indicate that the ability of casein, when fed at a high level, to counteract the symptoms of chronic selenium poisoning does not apply to all other proteins. When different proteins were used extreme differences in toxicity

were observed with seleniferous diets containing the same proportions of carbohydrate, fat, and protein. No satisfactory explanation of these results can be offered here. It seems doubtful that an explanation can be made on the basis of a deficiency in some amino acid, since edestin was selected because it is usually considered to be a rather 'complete' protein, i.e., one containing adequate amounts of the amino acids essential for normal growth. Perhaps the explanation of these discrepancies lies in the ease with which the various proteins are digested and absorbed from the intestinal tract; no studies were made in this respect.

Although the various high-protein diets had similar selenium concentrations and caloric values, the daily food intakes of the animals varied markedly. Animals on the casein and lactalbumin rations ingested about twice as much selenium per day as did those on other diets, yet this additional intake of the toxicant was more than counteracted by the increased protein consumption. Of these two proteins, lactalbumin was less consistent in offsetting the selenium toxicity.

Smith ('39) has observed that a high-casein diet containing 10 p.p.m. of selenium caused no impairment of growth, and affected but slightly the ability to reproduce and the macroscopic appearance of the liver and other organs. In his experiments the selenium was bound organically in seleniferous flour, whereas in the present study sodium selenite was employed. Figure 2 indicates that when inorganic selenium is present in the diet to the extent of 35 p.p.m., the growth is somewhat curtailed even when large amounts of casein are fed. Autopsies performed at the conclusion of the experimental periods showed striking pathological changes in the livers of many of these animals, including many of those which appeared normal and were rapidly gaining in weight at the time. The observations (Smith, '39) that female rats are more susceptible than males to liver injury and that dietary casein tends to prevent atrophy of the reproductive organs have been confirmed in the present study.

It would be interesting to know what the reaction of rats would be to seleniferous diets high in zein, hordein, oryzenin,

gliadin and glutenin, since these proteins occur naturally in the toxic grains from the seleniferous districts in the Great Plains region. An extension of this work using certain of these and other proteins is contemplated.

SUMMARY

Earlier observations on young white rats dealing with the protective action of high levels of casein against chronic selenium poisoning have been confirmed. Good growth results when as much as 35 p.p.m. of selenium, as sodium selenite, is incorporated in a diet containing 30% of casein. Only one rat, a female, died during the 8-week experimental period.

The ability of lactalbumin, gelatin, and edestin to render similar protective action to rats against ingested selenium (35 p.p.m.) has been tested. These proteins were all superimposed on a basal level of 6% casein in amounts sufficient to give 30% of protein in the diets.

Lactalbumin tends, as does casein, to counteract the toxic effects of dietary selenium, but it does not give as consistently good results as does casein.

Edestin and gelatin, when fed at high levels in seleniferous diets, exert no detoxifying action and are in all respects comparable to a diet containing only 6% of casein. Rats on these diets failed to grow and died in most cases within 32 days. None of these animals lived beyond 53 days.

Pathological changes have been described.

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A COMPARISON OF THE UTILIZATION BY GUINEA PIGS OF EQUIVALENT AMOUNTS OF ASCORBIC ACID (VITAMIN C) IN LEMON JUICE AND IN THE CRYSTALLINE FORM [1]

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The identification of vitamin C as ascorbic acid and the synthesis of this substance have led to the development of titration methods for the quantitative estimation of vitamin C in biological tissues and fluids. It also has made possible the study of the comparative utilization by the body of ascorbic acid as it occurs in the natural state in foods and of the crystalline form of the vitamin.

Jacobsen ('35) reported a lower concentration of ascorbic acid in the adrenals of guinea pigs receiving 20 mg. daily of crystalline ascorbic acid, than in the same organs of animals receiving cabbage ad libitum daily; the cabbage was stated "to contain something like 20 mg. of ascorbic acid per day."

Fox and Levy ('36) found that dehydroascorbic acid was effective as an antiscorbutic but that it was not as easily stored as the parent substance. They also reported that the vitamin C present in the green leaves of lucerne was comparable to orange juice in its availability to the guinea pig. However, their data showed that four animals fed for 2 months on a basal diet plus 5 ml. of orange juice equivalent to 2.5–3.0 mg. of ascorbic acid per day had an average store of 0.50 mg. of ascorbic acid per gram of adrenal tissue as compared with five animals fed for 3 months on a basal diet plus lucerne leaves

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equivalent to 3.2 mg. of ascorbic acid per day, which had an average store of only 0.32 mg. per gram of adrenals.

Giroud, Leblond and Ratsimamanga ('37) reported on the stores of ascorbic acid in the adrenal glands of guinea pigs receiving their supply of the vitamin from beet leaves, alfalfa, and cabbage leaves and from pure ascorbic acid. However, the ascorbic acid was given in solution in orange juice and the animals received a different basal diet from those receiving vegetable supplements; comparable levels of ascorbic acid were not fed in each group. The ascorbic acid of beet leaves was reported to be less well utilized than that of alfalfa and cabbage leaves; 6.6 mg. of ascorbic acid daily as beet leaves gave a storage of 0.448 mg. per gram of adrenal, while 5 mg. of ascorbic acid as alfalfa produced an adrenal store of 0.695 mg. per gram. Alfalfa leaves supplying 40 mg. of ascorbic acid daily gave an adrenal store of 1.069 mg. per gram while a 50 mg. level of pure ascorbic acid dissolved in orange juice produced only 1.055 mg. per gram.

Hawley, Daggs and Stephens ('37) reported that there was better retention of ascorbic acid in the tissues of guinea pigs when the vitamin was ingested in the natural form as cabbage, alfalfa hay, and orange juice than when taken in the form of crystalline vitamin C.

This investigation has been undertaken with the hope of throwing further light on the question of utilization of ascorbic acid from different sources, by making direct comparison of two forms of the vitamin fed at the same level to comparable animals.

EXPERIMENTAL

Two series of guinea pigs were used, and in each case, ascorbic acid in crystalline form [2] was compared with an equivalent amount of ascorbic acid in the form of lemon juice.

Comparisons have been made on the basis of weight gains, scurvy scores, and the ascorbic acid content of the blood and of the adrenal glands.

Series I. The guinea pigs were 6 to 8 weeks of age and weighed approximately 300 gm. All were shown to be healthy and actively growing at the beginning of the experiment. The vitamin C-free basal diet was the same as that described by Todhunter ('36). The animals, eighteen in number, were caged separately, and weighed every other day. They remained on the basal diet for 13 days and then were given the supplementary feedings for 20 days, and were killed on the twenty-first day.

One group of nine animals was given 1 ml. of lemon juice daily, fed directly into the mouth of the animal from a graduated glass syringe. The ascorbic acid content of the lemon juice was determined daily by titration with 2,6 dichlorophenolindophenol; a solution of pure ascorbic acid was then prepared of such concentration that 1 ml. of this solution was equivalent in ascorbic acid content to 1 ml. of lemon juice for that day. A second group of nine animals received daily 1 ml. of this ascorbic acid solution. The average daily intake for the group was 0.47 mg. of ascorbic acid in 1 ml. of lemon juice, and the same amount of the pure substance in aqueous solution.

Just before death, 1 ml. of blood was removed from the heart of the anesthetized animal. Preliminary studies showed that there was no change in level of blood ascorbic acid of animals kept under ether anesthetic for 7 minutes. The determination of ascorbic acid content of the blood was by the micro method of Farmer and Abt ('36).

The degree of scurvy was rated at death according to the scoring method of Sherman and co-workers ('22).

The adrenals were removed immediately, washed in saline solution, dried on filter paper, weighed, and ground with acid-washed sand and a 3% solution of metaphosphoric acid. After centrifuging, decanting and washing twice, the combined extracts from the adrenals were titrated with 2,6 dichlorophenol-

indophenol solution which had been standardized against pure ascorbic acid.

The results are summarized in table 1. All of the animals were of the same weight at the beginning, and the average

TABLE 1

Data of animals receiving daily 1 ml. lemon juice or an equivalent solution of ascorbic acid

GUINEA PIG NO.	BODY WEIGHT			SCURVY SCORE	ADRENAL WEIGHT	ASCORBIC ACID IN ADRENALS		ASCORBIC ACID PER 100 ML. PLASMA
	Initial	14-day period	Final			Total	Per gram	
	gm.	*gm.*	*gm.*		*gm.*	*mg.*	*mg.*	*mg.*
Lemon juice 1 ml. daily								
640 ♀	309	354	378	1	0.2438	0.023	0.094	0.23
602 ♂	293	339	414	1	0.1904	0.018	0.095	0.18
597 ♂	297	375	424	1	0.3023	0.024	0.079	0.16
679 ♀	285	303	327	7	0.2369	0.018	0.077	0.16
668 ♂	305	362	390	5	0.2201	0.016	0.073	0.10
669 ♂	308	356	394	1	0.2801	0.018	0.065	0.10
728 ♀	285	331	395	tr	0.3179	0.036	0.113	0.1?
745 ♀	290	313	331	7	0.1557	0.015	0.098	0.13
735 ♂	280	316	385	3	0.1891	0.021	0.112	0.14
Average	295	339	382		0.2374	0.021	0.090	0.15
S.D.					±0.0549	±0.006	±0.017	±0.04
Ascorbic acid solution 1 ml. daily								
607 ♀	291	279	294	4	0.2622	0.023	0.088	0.14
639 ♂	307	358	452	2	0.2743	0.034	0.124	0.21
615 ♂	294	352	406	2	0.2410	0.023	0.095	0.11
677 ♂	312	326	287	8	0.2221	0.017	0.076	0.14
678 ♀	290	318	390	6	0.2112	0.038	0.180	0.20
683 ♀	282	295	337	8	0.2772	0.038	0.139	0.19
747 ♂	285	313	352	6	0.1917	0.021	0.109	0.13
734 ♂	320	368	475	1	0.2689	0.038	0.141	0.07
730 ♂	287	274	362	6	0.1920	0.015	0.078	0.11
Average	296	320	372		0.2378	0.027	0.114	0.14
S.D.					±0.0347	±0.010	±0.035	±0.05

weight for each group was approximately the same at the end of the period. No difference was noted in either the average adrenal weights, or in the total ascorbic acid content of the adrenals of the two groups, and the ascorbic acid content of the blood plasma was the same. There was a wide variation

in the scurvy scores of individual animals in each group, but there were more low scores in the lemon juice group.

Series II. Since no difference in the growth, or body stores of ascorbic acid was found in the animals receiving such a low level of the vitamin, it was decided to see whether differences could be observed at a higher level of intake of the vitamin. Two milliliters of lemon juice were believed to be the amount which a young animal could reasonably consume at one time without introducing a possible error due to lack of absorption or intestinal destruction through delayed absorption. Therefore, the second series of animals received daily a total of 4 ml. of lemon juice fed in two portions.

Twelve animals were used in this series and they were comparable in age and weight to those of the first series. During the preliminary period when the guinea pigs were attaining the required weight of 300 gm., all animals received the basal diet plus spinach and carrots ad libitum. These animals were not made scorbutic first as in series I but, in an attempt to equalize the vitamin C reserves at the beginning of the experiment, all were kept for 1 day on the basal diet only, and then for 3 days, each received in addition 1 ml. of lemon juice daily. The animals were then divided in two groups: the first group received, daily, 4 ml. of lemon juice, 2 ml. being fed in the morning and 2 ml. at noon; the second group received 2 ml. of ascorbic acid solution in the morning and 2 ml. at noon, and this solution was equivalent in ascorbic acid content to the lemon juice. The daily feedings of the supplement were continued for 30 days.

The average ascorbic acid content of the 4 ml. of lemon juice which was fed daily during the experimental period was 1.80 mg. and therefore, the average intake of ascorbic acid from either source averaged 1.80 mg. daily for 30 days. At the end of the experimental period, the animals were killed, scored for scurvy and analyses of blood and adrenals were made as described for series I. The data for this group of animals are summarized in table 2. The animals receiving lemon juice showed no signs of scurvy, but four of the animals

receiving crystalline ascorbic acid had some macroscopic traces of hemorrhage in the joints or muscles. The ascorbic acid content of the blood plasma of both groups was the same and was higher than that of the animals in series I. The adrenal glands were smaller in the two groups of series II and this is in agreement with the findings of other investigators

TABLE 2

Data of animals receiving daily 4 ml. lemon juice or an equivalent solution of ascorbic acid

GUINEA PIG NO.	BODY WEIGHT		SCURVY SCORE	ADRENAL WEIGHT	ASCORBIC ACID IN ADRENALS		ASCORBIC ACID PER 100 ML. PLASMA
	Initial	Final			Total	Per gram	
	gm.	*gm.*		*gm.*	*mg.*	*mg.*	*mg.*
Lemon juice, 4 ml. daily							
753 ♂	300	491	0	0.1344	0.049	0.367	0.29
761 ♂	306	519	0	0.3851	0.117	0.303	0.22
785 ♀	300	323	0	0.1671	0.022	0.130	0.21
773 ♂	298	440	0	0.2194	0.050	0.227	0.19
755 ♂	293	287	0	0.1672	0.021	0.124	0.26
784 ♀	312	407	0	0.2114	0.046	0.217	0.21
Average	301	411		0.2141	0.051	0.228	0.23
S.D.				±0.0894	±0.035	±0.095	±0.04
Ascorbic acid solution, 4 ml. daily							
757 ♀	295	281	tr	0.2155	0.016	0.076	0.23
763 ♀	300	310	0	0.2375	0.030	0.128	0.17
767 ♂	306	560	0	0.1397	0.048	0.343	0.25
752 ♂	296	368	tr	0.1763	0.043	0.246	0.22
783 ♀	294	420	tr	0.3230	0.037	0.115	0.19
759 ♂	292	392	tr	0.2594	0.070	0.268	0.24
Average	299	388		0.2252	0.041	0.196	0.22
S.D.				±0.0643	±0.018	±0.104	±0.03

(Quick, '33; Hou, '34) that the adrenal gland becomes enlarged in scurvy. The amount of ascorbic acid stored in the adrenal gland was greater in both groups of animals of series II. The adrenals of the animals receiving 4 ml. of lemon juice showed a higher average content of ascorbic acid than those of the animals fed the equivalent ascorbic acid solution, but the difference is probably too small to be considered significant.

At the levels of ascorbic acid intake used in this study the lemon juice and crystalline ascorbic acid appear to be equally well utilized as a source of vitamin C when judged by the blood level and storage of ascorbic acid in the adrenal glands. However, the animals receiving crystalline ascorbic acid showed more evidence of hemorrhages and therefore, had higher scurvy scores than those receiving lemon juice. Bentsáth and co-workers ('36) reported that vitamin P was a factor in the maintenance of normal capillary resistance but Zilva ('37) failed to confirm this finding. The existence of vitamin P has yet to be conclusively demonstrated but Elmby and Warburg ('37) have found that ascorbic acid alone fails to cure the hemorrhagic condition of human scurvy.

The data presented in this paper, though not conclusive, do indicate the possibility that lemon juice may contain another factor concerned in the prevention of the hemorrhages which are characteristic of scurvy.

SUMMARY

Animals fed comparable amounts of ascorbic acid [3] in aqueous solution, and as lemon juice made similar gains in weight, but those receiving lemon juice had fewer hemorrhages when scored for scurvy.

The blood plasma levels were the same and there was no appreciable difference in the ascorbic acid content of the adrenals of each group.

The data indicate the possibility that lemon juice contains an additional factor which is concerned in the prevention of the hemorrhages characteristic of scurvy.

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A COMPARISON OF THE UTILIZATION BY COLLEGE WOMEN OF EQUIVALENT AMOUNTS OF ASCORBIC ACID (VITAMIN C) IN RED RASPBERRIES AND IN CRYSTALLINE FORM[1,2]

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In the preceding paper it was shown that vitamin C in the form of lemon juice or crystalline ascorbic acid was equally well utilized by guinea pigs when fed at levels of 0.5 mg. and 2 mg. daily and judged by weight gains, the storage of ascorbic acid in the adrenal glands, and the blood level of ascorbic acid.

The investigation here reported was undertaken to determine whether there was any measurable difference in the utilization by college women of ascorbic acid as it occurs naturally in red raspberries and in the crystalline form.

Ascorbic acid is not excreted in the urine in any appreciable amounts unless the requirements of the organism are fully met. Similarly, the blood plasma content of ascorbic acid falls almost immediately if the intake is inadequate, if there is incomplete absorption, or if there is destruction of the vitamin in the intestinal tract or other parts of the body. The technic used in this investigation, while it does not throw further light on the problem of what happens to ascorbic acid within the

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[1] Published as scientific paper no. 422, Agricultural Experiment Station, State College of Washington.

[2] This investigation is part of the regional project of the Northwest States on the ascorbic acid metabolism of college students.

[3] The data of this study are taken from a thesis submitted by Alva Fatzer in partial fulfillment of the requirements of the degree of master of science.

system, nevertheless, does serve as a measure of the comparative utilization of the vitamin from different sources; a fall in ascorbic acid content of either of the body fluids studied would indicate an intake inadequate to meet the metabolic needs of the organism.

Hawley and co-workers ('36) found that approximately the same amount of vitamin C was excreted in the urine when 100 mg. of ascorbic acid was ingested as orange juice or as crystalline ascorbic acid.

Elmby and Warburg ('37) reported that a total intake of 6 gm. of ascorbic acid failed to cure the hemorrhagic condition present in three patients with scurvy, but the juice of ten lemons daily for 10 days was therapeutically effective. They have suggested that some unknown substance, a co-vitamin, is required for the absorption and retention of ascorbic acid, and that this substance may either be part of some foodstuff or may be produced in the intestine under normal conditions.

McGovern, Gannon and Wright ('39) have also reported lack of success of oral intake of ascorbic acid as a cure for scurvy in some subjects unless the dose was extremely high.

EXPERIMENTAL

The urinary excretion and the blood plasma content of ascorbic acid were the criteria for studying the utilization of ascorbic acid.

Subjects

Seven college women, graduate students and faculty, served as subjects and gave full cooperation throughout the entire study. The age, height and weight of the subjects were as follows: A, 23 years, 162.0 cm., 51.26 kg.; B, 29 years, 161.0 cm., 51.71 kg.; C, 36 years, 161.3 cm., 62.60 kg.; D, 28 years, 154.0 cm., 49.90 kg.; E, 31 years, 157.5 cm., 61.69 kg.; F, 36 years, 165.1 cm., 60.33 kg.; G, 37 years, 173.5 cm., 66.68 kg.

Experimental period

The study was divided into four consecutive periods as follows:

Preliminary: Subjects ate their usual diets for 3 days.

Period I: Consisted of 3 days during which time the subjects remained on their usual diets plus the juice of five oranges each day. The orange juice was taken in two portions, at breakfast and at noon, and supplied approximately 200 mg. of ascorbic acid. At the end of this period the subjects were assumed to be saturated with vitamin C.

Period II: Subjects continued for 6 days on a basal diet supplying approximately 20 mg. ascorbic acid per day, supplemented with a daily portion of frozen red raspberries containing 40 mg. of ascorbic acid.

Period III: The usual diet was again resumed for 3 days with addition of orange juice as in period I, to bring the subject to a state of saturation.

Period IV was similar to period II except that the daily supplement was 40 mg. crystalline ascorbic acid.[4]

Basal diet

The basal diet used in this study was that recommended by Hauck ('38) and consisted of: 60 gm. American cheese, 100 gm. canned carrots plus 10 gm. juice, 60 gm. cooked dried prunes plus 10 gm. juice, 100 gm. canned pears plus 50 gm. juice, 100 gm. canned whole beets plus 10 gm. juice, 100 gm. ground beef, 60 gm. evaporated milk. The following foods were used ad libitum: Ry-Krisp, whole wheat cereal, eggs, rice, butter, nuts, sugar, whole wheat and white flour. The water intake was approximately equalized for all subjects and consisted of one cup of coffee at breakfast and five cups of water throughout the day at regular intervals. The canned foods were purchased in one lot for the entire study and the same brand was used throughout. Samples of the fruits, vegetables, milk and meat were analyzed for vitamin C by titration with 2,6 dichlorophenolindophenol. Assuming that the reducing value determined by titration was due solely to

vitamin C the portion of each food consumed daily contributed the following amounts of ascorbic acid: pears and juice 2.8 mg., beets and juice 11.1 mg., evaporated milk 0.7 mg., carrots and juice 1.6 mg., prunes 2.8 mg., beef 1.0 mg., giving a total intake of ascorbic acid from the basal diet of 20 mg. per day. The basal diet was estimated from tables of food composition to supply: 47.7 gm. protein, 0.863 gm. calcium, 0.983 gm. phosphorus, 9.78 mg. iron. Adequate calories were obtained from the ad libitum foods, and the liberal use of butter and whole grain cereals probably supplied sufficient vitamins A and B; the evaporated milk was irradiated.

All meals were prepared and eaten in the college diet kitchen.

Dietary supplements

Red raspberries. The red raspberries were of the Antwerp variety grown at the Western Washington Experiment Station,[5] frozen-packed immediately after harvesting and stored at 0° F. The berries were packed in no. 2 enamel-lined cans; as required, cans were opened and the contents weighed out so that each portion contained 40 mg. ascorbic acid. The berries were eaten at breakfast in the partially thawed state.

The ascorbic acid content of the berries was determined by titration [6] with 2,6 dichlorophenolindophenol according to the method of McHenry and Graham ('35).

Ascorbic acid. In period IV the supplement of 40 mg. ascorbic acid was taken in the form of the crystalline substance dissolved in a small amount of distilled water. The ascorbic acid solution was taken at breakfast time.

Urinary collection and analysis

Urine was collected at the end of the first 6 hours of the morning excretion and for the remaining 18 hours. Collection

was made in 1 quart jars containing the following amounts of a preservative recommended by Sendroy ('37) : 75 ml. of 5 N. sulphuric acid, 0.75 ml. 8-hydroxyquinoline (1.45 gm. in 100 ml. of ethyl alcohol) and 5 ml. toluene.

Recovery tests on samples of urine with this preservative showed that it permitted negligible destruction of ascorbic acid during the 24-hour period. The ascorbic acid content of the urine was determined by titration with 2,6 dichlorophenol-indophenol which had been standardized against a solution of pure ascorbic acid. The dye solution was of such strength that 2 to 5 ml. were required for the titration of aliquots of 10 or 20 ml. of urine, depending on the concentration of ascorbic acid in the urine. Titrations were completed in less than 2 minutes and the end point was taken as a faint pink color lasting 10 seconds.

Blood analysis

Blood ascorbic acid was determined by the micro-method of Farmer and Abt ('36). Blood samples were taken from a finger prick while the subject was in the post-absorptive state and analyses were made at the end of the preliminary period and the beginning and end of period II and IV. Subjects A and B were tested for kidney function by injection of phenol-sulphonaphthalein and were found to be normal.

RESULTS AND DISCUSSION

In the preliminary period on their usual diets the subjects showed a variable excretion of ascorbic acid, the lowest level being 9 mg. for subject B as seen from table 1. All subjects immediately gave a marked rise in excretion the first day that oranges were added which would indicate that even those subjects with a previously low excretion of vitamin C were yet in a good state of vitamin C nutrition. The blood plasma levels confirmed this (table 2), subject A showing 0.91 mg. per 100 ml. of plasma and all the others above 1 mg. per 100 ml.; a level of 0.8 mg. or higher is generally accepted as indicating

a satisfactory state of vitamin C nutrition. Therefore the level of urinary excretion of subjects on their usual diets cannot alone serve as an indicator of vitamin C 'saturation.' In both periods II and III when orange juice was added to the basal diet the subjects were 'saturated' with vitamin C as judged by the increased urinary excretion and blood plasma content

TABLE 1

Excretion of ascorbic acid by subjects on basal diet plus 40 mg. of crystalline ascorbic acid and an equivalent amount from red raspberries

DIETARY PERIOD	MILLIGRAMS OF ASCORBIC ACID EXCRETED PER 24 HOURS FOR SUBJECTS:							
	A	B_1	B_2	C	D	E	F	G
Usual [1]	70	9	21	28	98	63	28	103
Usual + oranges [1]	271	160	127	267	205	241	316	262
Basal + raspberries								
1st day	87	22	21	101	46	74	73	47
2nd day	77	36	22	53	40	44	40	52
3rd day	59	35	18	44	28	40	26	22
4th day	45	10	5	39	40	14	18	20
5th day	31	29	15	22	29	22	16	26
6th day	32	13	8	18	22	25	13	16
Usual + oranges [1]	271	154	175	260	211	220	275	214
Basal + ascorbic acid								
1st day	58	43	29	83	68	45	72	27
2nd day	40	17	25	44	43	34	47	16
3rd day	34	18	16	24	24	20	26	22
4th day	11	9	6	18	22	14	22	21
5th day	28	10	10	16	15	17	19	14
6th day	16	13	13	16	25	11	12	19

[1] Ascorbic acid value for the last day only of each of these periods is given.

TABLE 2

Ascorbic acid content of blood plasma of subjects on the last day of each dietary period

DIETARY PERIOD	MILLIGRAMS OF ASCORBIC ACID PER 100 ML. BLOOD PLASMA OF SUBJECTS:							
	A	B_1	B_2	C	D	E	F	G
Usual	0.91	1.03	0.99	0.86	1.65	1.39	1.66	1.45
Usual + orange juice	1.78	1.86	1.83	1.89	1.68	1.69	1.94	1.69
Basal + raspberries	1.45	0.92	1.17	1.42	1.26	1.10	1.13	1.19
Usual + oranges	2.14	2.27	1.68	1.56	1.56	1.79	1.80	1.47
Basal + ascorbic acid	1.80	1.46	1.09	1.14	1.22	1.11	1.14	1.10

of ascorbic acid. All were, therefore, in a comparable state at the beginning of each of the two test periods of receiving the basal diet plus 40 mg. ascorbic acid and the equivalent amount of frozen packed red raspberries.

During each of these test periods, the urinary excretion fell for the first 3 or 4 days and then became more constant, though there was considerable fluctuation for each individual even on this uniform daily intake. This daily fluctuation makes it somewhat difficult to compare the excretion during the two periods. Sendroy and Schultz ('36) proposed a utilization index, I, for comparing the state of ascorbic acid nutrition of various individuals. This index takes into consideration the body weight and age of the subject. Sendroy reported the normal utilization index to vary only within average limits of 67.5 ± 5.5 and he arrived at this figure from a study of the output of subjects receiving 250 mg. ascorbic acid daily for 7 days. Since the two test periods for each subject in this investigation were exactly the same with regard to vitamin C intake, the only variable being the source of vitamin C, it was considered justifiable to use Sendroy's formula for comparing these data, even though the previous histories were different and the level of intake was lower than Sendroy had used. The smaller the difference between output and intake of vitamin C, the better the utilization and the smaller the resultant figure for the utilization index.

The utilization indexes are summarized in table 3 and show for all subjects, except A and C, a comparable value for the two test periods. Subjects A and C each had a lower index during the period when raspberries were the supplement, indicating that there was probably better utilization of the ascorbic acid of the berries by these two subjects.

For each subject, the raspberry supplement period preceded that when ascorbic acid was used and it was considered possible that there might be some cumulative effect of ascorbic acid intake in raising the blood level at the end of the experiment since A and B, the first two subjects on the study, showed a higher blood value (table 2) at the end of the ascorbic acid

period than at the end of the raspberry period. Subject B therefore repeated the experiment reversing the order of taking the supplements in the two test periods; this had no influence on the results as is shown in tables 1 and 2, where the repeat experiment for this subject is indicated as B_2. The utilization index was also unchanged in the second experiment for B_2 as shown in table 3.

TABLE 3

Index of utilization [1] of crystalline ascorbic acid and an equivalent amount of vitamin C from red raspberries

SUB-JECTS	DIETARY SUPPLEMENT	ASCORBIC ACID			BODY WEIGHT	UTILIZA-TION CO-EFFICIENT	$\sqrt{\text{AGE}}$	UTILIZA-TION INDEX
		In-take	Out-put	Differ-ence				
		mg.	mg.	mg.	kg.			
A	Ascorbic acid	360	188	172	51.26	3.4	4.8	16.2
	Raspberries	360	330	30		0.6		2.8
B	(1) Ascorbic acid	360	110	250	51.71	4.8	5.4	26.1
	Raspberries	360	144	216		4.2		22.5
	(2) Ascorbic acid	360	99	261		5.0		27.3
	Raspberries	360	90	270		5.2		28.3
C	Ascorbic acid	360	202	158	62.60	2.5	6.0	15.2
	Raspberries	360	276	84		1.3		8.0
D	Ascorbic acid	360	197	163	49.90	3.3	5.3	17.3
	Raspberries	360	206	154		3.1		16.5
E	Ascorbic acid	360	140	216	61.69	3.6	5.6	19.9
	Raspberries	360	220	140		2.3		12.8
F	Ascorbic acid	360	197	163	60.33	2.7	6.0	16.2
	Raspberries	360	186	174		2.9		17.3
G	Ascorbic acid	360	119	241	66.68	3.6	6.1	22.0
	Raspberries	360	182	178		2.7		16.2

[1] Index of utilization was calculated from Sendroy and Schultz, J. Clin. Invest., vol. 15, p. 369, 1936.

The data indicate that 40 mg. of ascorbic acid obtained from red raspberries were fully as well utilized as the same amount of the vitamin in crystalline form.

Short period collections of urine rather than the total 24-hour amount have been suggested by some workers as an adequate indication of whether there is a marked response to the test dose. Harris and Abbasy ('37) analyzed a 3-hour

morning specimen, whereas other workers (Goldsmith and Ellinger, '39) based their study on a 6-hour collection.

In this investigation 6-hour collections of urine were made daily for each subject; the bladder was emptied on rising in the morning and the urine for the succeeding 6 hours was collected and the vitamin C content determined immediately by titration. The per cent of the total 24-hour excretion of ascorbic acid which was eliminated in the first 6 hours was then calculated for each subject. The per cent excreted in this period varied for the different subjects and showed a wide variation for the same subject. This is illustrated by the following data for two subjects, expressed as per cent of the 24-hour excretion for the different dietary periods: subject A, preliminary period, 37, 18, 33; period I, 28, 35; period II, 47, 55, 60, 61, 45; period III, 29, 34, 52 and period IV, 39, 44, 59, 25, 33; subject D, preliminary period, 32, 25, 43; period I, 47, 44; period II, 62, 39, 54, 47, 50; period III, 27, 40, 50; period IV, 66, 56, 45, 50, 52.

The other subjects showed a similar range of excretion values; the lowest value was 6% for subject C who, on another day, excreted 61% of the total in the first 6 hours. With such variations it is to be questioned whether the 6-hour urine specimen can give reliable information regarding the total daily excretion of ascorbic acid.

SUMMARY

Seven college women served as subjects for a study of the comparative utilization of ascorbic acid in red raspberries and in the crystalline form of the vitamin. The subjects were 'saturated' with vitamin C at the beginning of each test period and consumed weighed amounts of the same basal diet throughout.

The urinary excretion and blood plasma level of ascorbic acid were similar for the same subject when receiving vitamin C from red raspberries and from crystalline ascorbic acid. There was considerable variation among subjects with respect

to their blood level and excretion of ascorbic acid under comparable conditions.

The utilization index as calculated from Sendroy's formula showed the ascorbic acid of red raspberries to be as well utilized as crystalline ascorbic acid.

The per cent of the total 24-hour excretion of ascorbic acid eliminated during the first 6 hours varied widely in the same individual and for the different subjects.

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GROWTH OF RATS ON HIGH FAT AND LOW FAT DIETS, DEFICIENT IN THE ESSENTIAL UNSATURATED FATTY ACIDS [1]

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FIVE FIGURES

During the past several years it has been shown beyond doubt that the rigorous exclusion of fat from the diet of the rat results in a typical deficiency disease, of which the outstanding symptom is subnormal growth. Since the impairment of growth can be prevented or cured only by the feeding of small amounts of either linoleic, linolenic, arachidonic, or docosahexaenoic acid, it has been concluded that these more highly unsaturated fatty acids, essential for normal growth and health, cannot be synthesized by the rat—at least in appreciable amounts—and are therefore essential dietary constituents (Hume et al., '38).

In the course of earlier studies with elaidic acid (Sinclair, '35), some young rats at weaning age were fed on a diet very rich in elaidin [3] (diet 290, table 1). This diet was identical in composition with our standard low fat diet (diet 3, table 1) save for the replacement of the sucrose by an isocaloric amount of the fat elaidin. The contrast between the growth of the rats

[1] Part of this work was presented before the American Society of Biological Chemists at Washington, D. C., in March, 1936.

[2] A considerable part of this work was done in the Department of Biochemistry and Pharmacology, University of Rochester Medical School, Rochester, N. Y.

[3] The term 'elaidin' is used to designate the solid fat prepared from olive oil by treating with N_2O_3 and crystallizing several times from acetone. On the basis of their iodine number, the fatty acids of elaidin are assumed to consist of about 85% elaidic acid and 15% saturated fatty acids.

on the high elaidin diet and that typical of the high carbo-
hydrate diet was very striking. On the low fat diet (fig. 1, curve
1) growth, though subnormal, is maintained for several months
and, when it finally stops, the subnormal weight is maintained
more or less constant for months before the final decline sets
in. On the high elaidin diet, on the other hand (fig. 1, curve 3),
growth proceeded for only 4 to 6 weeks and stopped, rather
abruptly, when the average weight was only about 100 gm.[4]
This weight was usually maintained for several weeks and then
a gradual decline set in, ending in the death of the animal at
an age of about 4 months.

At this same time some young rats had been started at
weaning age on a high elaidin diet (290-C, table 1) differing
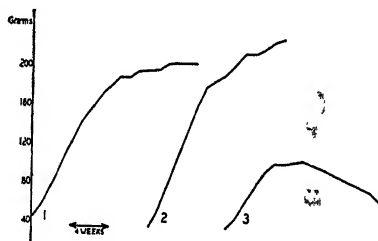
Fig. 1   Curve 1—Composite weight record of sixteen male rats fed on a high
carbohydrate low fat diet (no. 3, table 1). Curve 2—Composite weight record of
seven male rats fed on diet 290-C, elaidin (table 1). Curve 3—Composite weight
record of five male rats fed on diet 290, elaidin (table 1).

from diet 290 in that about 5% of the elaidin was replaced
by a 1:1 mixture of cod liver and corn oils. As shown by
curve 2, figure 1, the growth of rats on this latter high fat
diet was very good indeed. Furthermore, several rats were
transferred from the straight elaidin diet to diet 290-C after
there had been a considerable loss in weight, and death seemed
imminent. Curve 1, figure 2, which is quite typical, shows that
there is a prompt resumption of growth. Since cod liver and

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[4] The maximum weight attained was found to depend largely on the weight
of the rat when placed on the elaidin diet. Some animals, weighing 48 gm.
when 21 days old, reached a weight of 146 gm. The amount and quality of
the depot fat in the young rat appear to be of primary importance.

corn oils are rich in the essential unsaturated fatty acids, one is led to conclude that the very poor growth of the rats on the straight elaidin diet is due to a severe deficiency of those fatty acids which are essential for growth and health.

Naturally, the question arises as to the reason for the much better growth on the high carbohydrate diet. Up to the present time it has been assumed that the unsaturated fatty acids needed for the growth of rats on high carbohydrate low fat diets have been derived exclusively from two sources:

TABLE 1

Percentage composition of diets

INGREDIENTS	PER 100 GM.		
	Diet 3	Diet 290	Diet 290-C
Casein [1]	17.1	28.1	28.1
Sucrose	70.3		
Salt mixture [2]	3.8	6.3	6.3
Yeast	8.8	14.4	14.4
Elaidin		51.2	48.4
Corn oil			1.4
Cod liver oil [3]			1.4

[1] Neither the casein nor the yeast was extracted with fat solvents. By analysis, the casein in diet 3 was found to supply 340 mg. and the yeast 505 mg. of total fatty material per 100 gm. of diet.

[2] McCollum, E. V., and Simmonds, N., J. Biol. Chem., vol. 33, p. 63 (1918).

[3] Vitamins A and D were supplied to rats on diet 3 or 290 in the following ways: (1) In the earlier experiments, the rats received daily the unsaponifiable material prepared from 150 mg. of cod liver oil, dissolved in 2 drops of mineral oil; (2) more recently, the rats were given 1 mg. of percomorph oil dissolved in 2 drops of mineral oil or 2 drops of melted elaidin. This method is more convenient and gives growth records indistinguishable from the first.

the small amount of fat in the diet; and the store of fat laid down in the depots during the suckling period. In the case of the rats fed on the high carbohydrate and high elaidin diets, the amount of unsaturated fatty acids derived from these two sources must be about the same. Nevertheless, the deficiency of essential fatty acids seems to be much less severe in rats on a high carbohydrate diet than in those on the high elaidin diet.

There appear to be two possible explanations. One is that the rats on the high carbohydrate diet are able to synthesize certain of the fatty acids needed for growth, even though the amount or kind—or both—synthesized is insufficient to maintain normal growth and health. In the case of the rats on the high fat diet, such synthesis of fatty acids from carbohydrate would, of course, be completely suppressed. They would therefore be entirely dependent upon the diet and pre-existing stores for the supply of essential fatty acids. The second possibility is that the requirement of essential fatty acids is greater on a high fat than on a low fat diet, either because of the intensity of fat and, therefore, of phospholipid metabolism or because of a purely physical 'masking' of the essential unsaturated fatty acids by the large amount of elaidic acid simultaneously being absorbed and transported. In either case, one would have to assume that the supply of unsaturated fatty acids in the food and stores is sufficient to permit considerable growth on the high carbohydrate diet but is quite inadequate for rats on the high fat diet.

RESTORATION OF GROWTH

After growth had stopped, a number of rats on the high elaidin diet were given daily, by mouth, graded amounts of corn oil. Representative records are given in figure 2 (curves 2, 3, 4 and 5). It will be seen that 1 drop (20 mg.) of corn oil, or of percomorph oil,[5] is insufficient to induce a significant gain in weight although it does prevent the decline which otherwise would probably have set in. On the other hand, 5 drops (100 mg.) of corn oil induce a pronounced increase in weight; the effects of 10 and 20 drops were, in general, still better. In every case, however, growth stopped when the animal was still well below the normal adult weight.

An even more striking resumption of growth takes place if the high elaidin diet is replaced by one rich in carbohydrate.

[5] Mead Johnson & Co. Oleum percomorphum.

The five growth records in figure 3, representative of fifteen in all and including the poorest, illustrate the rapid gain in weight which promptly sets in when the elaidin diet is
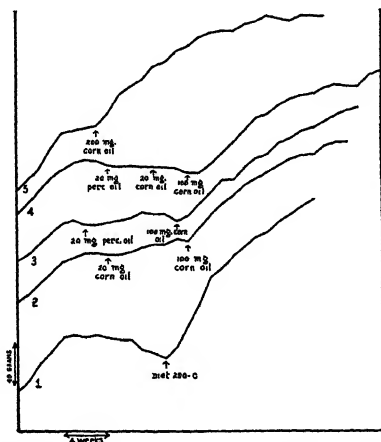
Fig. 2 Curve 1—Weight record of a male rat fed on diet 290, elaidin and then transferred to diet 290-C, elaidin. Curves 2 to 5—Weight records of rats fed on diet 290, elaidin supplemented by percomorph oil or corn oil.
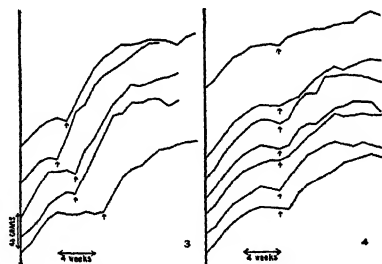
Fig. 3 Weight records of rats fed on diet 290, elaidin and then changed to diet 3 (table 1).

Fig. 4 Weight records of rats fed on diet 290, elaidin and then fed on a high carbohydrate diet very low in fat (diet 6 b).

replaced by diet 3. The only change in the diet is the replacement of the fat by sucrose. After a gain of 80 gm. or more, resulting in some cases in a doubling of the weight in 4 weeks, the growth ceases once more when the weight is about

the same as that characteristic of rats fed from weaning age on the same high carbohydrate diet.

When these results were first obtained, it was felt that the only satisfactory interpretation was that the resumption of growth on the carbohydrate diet had been made possible by the synthesis of the necessary fatty acids from carbohydrate (Sinclair, '36). Later it was recognized that the restored growth could conceivably be attributed to the small amount of essential acids, present in the casein and yeast fat, which, though quite unable to support growth on the elaidin diet, became effective when the fat was replaced by sucrose.

It was decided therefore to repeat the experiments, using a diet which, though not strictly fat-free, contained very much less fat than diet 3. The composition of this diet (no. 6 b) was as follows: Vitamin-free casein,[6] 16.6%; sucrose, 75.6%; salt mixture, 3.8%; Harris water-soluble yeast concentrate,[7] 4%. Vitamins A and D were supplied by giving daily 2 drops of propylene glycol carrying $50\,\gamma$ of carotene[8] and $0.2\,\gamma$ of calciferol.[9]

As in the earlier experiments, young rats were fed on the elaidin-containing diet 290 until growth had ceased and the weight had remained constant for several weeks.[10] The diet was then changed to 6 b. The weight records of seven rats are shown in figure 4. In every case there was a resumption of growth. The gain in weight ranged from 34 gm. to 60 gm., the maximum gain in a single week being 19 gm.

Now, analysis of the Harris yeast concentrate, the only possible source of essential fatty acids in diet 6 b, showed that 10 gm. contained 28 mg. of ether-soluble and petroleum ether-soluble material. One may assume that perhaps 25% of these fatty acids consists of linoleic acid (Newman and

[6] Obtained from S. M. A. Corporation, Cleveland, O.

[7] Obtained from Harris Laboratories, Tuckahoe, N. Y.

[8] Kindly donated by Hoffmann-LaRoche, Ltd.

[9] Kindly supplied by Dr. C. E. Bills of Mead Johnson & Co.

[10] In all cases, the rats were kept in individual cages with bottoms made of wire screen ($\frac{1}{2}$ inch mesh).

Anderson, '33). On the basis of a daily consumption of 10 gm. of ration per day, each rat would thus obtain in the diet 1.1 mg. of fatty acid and about 0.3 mg. of linoleic acid.

It is probably safe to assume, on the basis of existing data (Sinclair, '30), that an increase of 10 gm. in the body weight of a rat involves an increase of about 100 mg. in the phospholipid fatty acid content of the body. Consequently it may be calculated that a rat which increased its weight by 19 gm. in a week probably added about 190 mg. to its phospholipid fatty acids and, during the same period, took in not more than 8 mg. of fatty acids with its food. Clearly therefore, the daily increment in phospholipid fatty acids is several times greater than the amount of fatty acid that could possibly have been obtained from the diet. Because of the nature of the previous diet, the depot fat of these rats can conceivably be a source only of some of the fully saturated acids, of elaidic acid, and possibly of some oleic acid. From what is known about the phospholipids, it is very unlikely that these fatty acids, together with the trace of linoleic obtained from the diet, would suffice for phospholipid synthesis. One is led to conclude therefore that the rats on the high carbohydrate diet synthesized the bulk of the fatty acids needed for growth. Whether or not even the limited growth which did occur was made possible only by reason of the trace of fat present in the diet is, of course, a question which can only be settled by feeding a strictly fat free diet.

On comparison of the growth curves in figures 4 and 3, it is evident that the gain in weight by rats fed on the more highly purified diet 6 b was inferior to that of most of the rats on diet 3. To rule out the possibility of there being an insufficiency of thiamin and lactoflavin, 10 γ of each of these two factors were fed daily for several weeks to the rats on diet 6 b. No effect on growth was apparent. It seems likely therefore that the difference is due to the higher fat content of diet 3. This is borne out by the growth records in figure 5 which show that either the replacement of diet 6—similar to 6 b

except for the use of non-extracted casein—by diet 3 or the feeding of 50 mg. of yeast fat caused a resumption of growth.

An increase or a decrease in body weight has been the only feature dealt with in this paper. It needs to be emphasized that, even though the rats gained considerably in weight on the high carbohydrate diet, their condition did not improve to an extent at all comparable to that of the animals fed a little corn oil in addition to the elaidin diet. Indeed, soon after growth had ceased once more, the weight began to decline and, within a short time, severe hematuria and other symptoms developed.
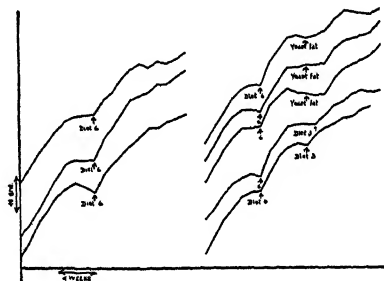
Fig. 5   Weight records of rats fed on diet 290, elaidin until growth had ceased and then transferred to a high carbohydrate diet low in fat (diet 6). When growth had stopped once more, some were transferred to diet 3; others were fed 50 mg. of yeast fat.

DISCUSSION

The findings presented in this paper can best be explained on the basis of the assumption that the rat is capable of synthesizing from carbohydrate the bulk of the fatty acids needed for growth. On the high elaidin diet, the supply of essential fatty acids derived from the pre-existing fat stores is sufficient to permit moderately good growth for a time; the greater the supply, the better the growth. When the supply is exhausted, growth stops because the small amount of essential fatty acid present in the diet is quite inadequate. If sufficient corn oil is fed, growth is restored. Twenty milligrams of corn oil is however insufficient, even though, on a high carbo-

hydrate diet, such an amount, judging from other evidence (Mackenzie et al., '39) permits excellent growth. The fatty acids synthesized from carbohydrate make up the deficiency. On replacing the elaidin of the diet by sucrose, after growth has stopped, synthesis of fatty acids sets in and these fatty acids, at least with the trace present in the diet, are sufficient to permit rapid and extensive growth. If the rat is placed on the high carbohydrate diet at weaning age, the synthesized fatty acids, together with those in the depots and in the diet, permit growth up to about 70% of the average normal weight. Normal growth and health can, however, only be secured by feeding certain of the unsaturated fatty acids.

On the other hand, it is entirely possible that an increase in the requirement of essential unsaturated fatty acids as a result of the very high fat diet is, in part at least, responsible for the poor growth and early decline of rats on the elaidin diet and for most of the observations reported above.

Regardless of which is the correct explanation, it ought to be possible to duplicate all of the results presented in this paper with some fat other than elaidin, provided it is totally free from the essential unsaturated fatty acids. Some experiments were carried out with hydrogenated coconut oil. In general, the results obtained were the same as those above. Evans and Lepkovsky ('32) some years ago reported very poor growth, comparable to that on elaidin, on diets rich in the glycerides of the completely saturated acids of coconut oil. There is no reason to believe that the growth of rats on the diet rich in elaidin is in any sense characteristic of that fat alone.

SUMMARY

On a diet of casein, salt mixture, dried yeast and the fat, elaidin, supplemented with vitamins A and D, rats cease growing when only about 100 gm. in weight. After several weeks at constant weight they go into a decline and die. Since growth and health are readily restored by feeding a little corn oil, the impairment in growth is attributed to a severe deficiency of the essential unsaturated fatty acids.

Replacement of the elaidin by sucrose also results in a rapid and extensive gain in weight. A similar, though smaller, gain occurs when the elaidin diet is replaced by one rich in carbohydrate and very poor in fat.

It is concluded that the better growth of rats on a high carbohydrate than on a high fat diet, both equally poor in essential fatty acids, is due, in part at least, to the synthesis of the fatty acids necessary for growth. An increase in the requirement of essential fatty acids by rats on a high fat diet may also contribute to the poor growth obtained.

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THE INDIGESTIBLE CARBOHYDRATES OF FEEDS

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The indigestible carbohydrate portion of feeds, especially of those referred to as roughages, has always been of considerable interest to nutrition workers. For over 75 years this indigestible residue has been referred to as crude fiber. The official A.O.A.C. method for its determination, known as the Weede method, has always been recognized as purely empirical, having little correlation with the residues from the true digestive processes, and failing to give a true conception of the composition of the residue. Therefore, we have no way of estimating the relative digestibility of the quantity of crude fiber reported, as the chemical makeup of this material may be quite different in samples from various plants, and the utilization entirely different depending upon the lignin-cellulose ratio. These difficulties have always been admitted but only in recent years has any systematic attempt been made to break down this material by more accurate methods of analysis for lignin, hemicellulose, and cellulose separately. A comprehensive study made during the past 2 years in this laboratory has demonstrated that none of the methods is entirely satisfactory, and, also, that one cannot expect to recover 100% of the original residue by purely empirical procedures.

It is much easier to cite reasons for these discrepancies than to offer remedies. In the first place, although numerous and extensive studies have been made, the true structure and properties of cellulose, lignin, or hemicellulose are not known,

and, therefore, only empirical methods are used in the determinations. Furthermore, the nature of the balance of the feed may affect profoundly the results obtained. These facts are suggested in the reports of many investigators. The excellent reviews as well as the original work of Norman ('34 and '35), and of Norman and Jenkins ('34) demonstrate clearly the difficulties presented.

When one attempts to recover the various fractions from a single sample, the task becomes still more difficult. Even the methods used are open to criticism in certain instances. In the method of Williams and Olmsted ('35) the use of an alkaline-enzyme digest results in the loss of some of the hemicellulose, and, in certain cases, separate determinations must be made for its complete recovery. The use of 60% H_2SO_4, in the light of the work of many investigators, notably Ritter, Seborg and Mitchell ('32), and Sherrard and Harris ('32) does not give as good results as a 70 to 72% solution. Under the conditions outlined this procedure is open to criticism. First, with 60% sulfuric acid it is doubtful if all the cellulose is entirely removed; second, an investigation of the literature does not present evidence that uronic acid is converted quantitatively into pentoses, as stated; and third, there is evidence that pentoses would be partly destroyed in the long acid hydrolysis.

In the Crampton and Maynard method ('38), on the other hand, the use of formaldehyde in the determination of lignin is not accepted by many lignin chemists due to the fact that formaldehyde condensation products must surely yield a high lignin result, and no measure of the hemicellulose fraction is presented. Although no method can be entirely quantitative until more fundamental methods are perfected, nevertheless, the rather empirical procedure as outlined does present a much more complete picture of the true composition of feeds than the crude fiber as ordinarily reported.

In view of these considerations, the modification of the Williams and Olmsted procedure has been used in analyzing many common cereal grains and forage crops as well as feces samples, and has proved to be the most acceptable in com-

parative tests. In digestibility studies attempts were made to ascertain if certain fractions of this material might be more digestible than others by comparing the relative proportions as the test substance passed through the intestinal tract of sheep and cattle. The ratio between the quantities of lignin, cellulose, and hemicellulose might be quite a changeable one if hemicellulose proved to be more digestible than lignin, and the so-called crude fiber content of certain feeds more desirable than an equal amount in plants having a different composition.

EXPERIMENTAL PART

Samples of cereal grains, seeds, hays, prairie grasses, and mixed feeds produced and commonly used in this state were chosen from samples being analyzed in this station. The samples were dried, ground in a Wiley mill, and further powdered in a Merker mill until they would completely pass through a 60-mesh sieve. They were then redried, and 0.5 gm. samples weighed in duplicate. The method of analysis was a somewhat modified form of the Williams-Olmsted procedure, and is briefly described as follows:

The sample was first extracted 16 hours with ether, dried, and transferred to a 250 ml. Erlenmeyer flask, and covered with a 50 ml. beaker. Twenty-five milliliters of water were added, and the flask and contents sterilized for 2 hours at 20 pounds pressure. The flasks were cooled, and the enzyme-digest mixture added which consisted of 20 ml. of bile buffer, 5 ml. of the pancreatin-sodium chloride solution, and a few drops of toluene. The flasks were transferred to a constant temperature water bath controlled at 45°C., and left for 72 hours with frequent shaking. At the end of this period the contents of the flasks were filtered through a silk bolting cloth, washed with hot alcohol, benzene, alcohol, and ether. The residue was then transferred to the original flask, and treated with 20 ml. of cold 70% sulfuric acid, and kept with frequent shaking for 16 hours at 4 to 10°C., as suggested in the procedure of Sherrard and Harris ('32). The residue and acid

were then transferred to 1000 ml. tall, lipless beakers, treated with 450 ml. of water, and boiled for 3 hours under condensers, and at once filtered with suction through RA 98, 25 ml. alundum crucibles. The filtrate was then transferred to 500 ml. volumetric flasks. The alundum crucibles containing the lignin were first washed very thoroughly with hot water to remove acids and sugars, then with hot water, alcohol, benzene, and ether, then dried, weighed, ashed, and reweighed, and the loss of weight calculated as lignin. The filtrate was exactly neutralized with 50% NaOH, and diluted to 500 ml. Five milliliter aliquots were used to determine the reducing sugars, and similar quantities of the samples which had been previously fermented with well-washed yeast were used for non-fermentable sugars following the original technique. From these determinations cellulose and hemicellulose were calculated as directed. The reducing sugar values were determined by comparing the titrations with curves which had been prepared from known sugars dissolved in similar solvents. There is nothing forbidding about the procedure. It is long and requires attention at definite intervals. Great care, skill, and attention as to the exact pH, time, and temperature were found to be necessary; however, with sufficient experience it can become a regular routine procedure, and can easily be carried out by any experienced technician. For the preparation of reagents and details of procedure one is referred to the Williams-Olmsted article ('35), and for questions concerning the sugar determination and preparation of sugar curves reference is made to the original Shaffer-Somogyi method ('33). Samples were also analyzed for crude fiber by the usual A.O.A.C. methods. The data for a number of common Oklahoma hays and grasses are recorded in table 1, and similar data for some common food concentrates are given in table 2. These data demonstrate that no fixed ratio exists between either the amounts of lignin, cellulose, and hemicellulose or crude fiber and the total indigestible residue of cereal grains.

STUDY OF INTESTINAL CONTENTS

A further purpose of this study was to determine if crude fiber from various sources was equally well utilized by animals; that is, to determine if a sample composed largely of hemicellulose or cellulose might have a higher coefficient of digestibility than one composed largely of lignin. It was possible to secure these data, as coefficients of digestibility of various feeds were being determined by this and cooperating

TABLE 1

Lignin, cellulose, and hemicellulose content of hay and related plant products. Milligrams per gram sample

SAMPLE	CRUDE FIBER	LIGNIN	HEMI-CELLULOSE	CELLULOSE	TOTAL IN-DIGESTIBLE RESIDUE
Alfalfa hay	246	180	112	153	445
Alfalfa leaf meal	166	129	79	131	339
Bermuda grass	254	111	158	192	461
Johnson grass	329	142	91	88	321
Little blue stem	324	199	156	152	507
Switch grass	320	217	139	102	458
Atlas sorgo	189	162	94	133	389
Clover hay	213	218	49	62	329
Sweet clover	305	209	99	105	413
Dwarf yellow milo	206	153	96	69	318
Millet hay	276	179	121	109	409
Mung bean hay	220	163	95	92	350
Soy bean hay	151	330	158	125	613
Wheat straw	364	310	146	115	571
Timothy hay	303	155	188	169	512

departments at the same time. Rats, chickens, sheep, and cattle were used in these experiments. The data for rats and sheep are presented. The usual methods of procedure for metabolism experiments were followed by the use of specially constructed feeders and cages with perforated floors which permitted the measurement of the weight of feed and excreta daily. These tests have been repeated a number of times, and the averages of a series of analyses of the data for digestibility coefficients of lignin, cellulose, and hemicellulose are

TABLE 2

Lignin, cellulose, and hemicellulose content of grains and seed concentrates.
Milligrams per gram sample

SAMPLE	CRUDE FIBER	LIGNIN	HEMI-CELLULOSE	CELLULOSE	TOTAL IN-DIGESTIBLE RESIDUE
Barley	54	68	59	26	154
Buck wheat	105	139	92	42	273
Broom corn	84	83	38	31	152
Yellow corn	21	23	49	45	117
Flint corn	20	73	62	37	172
Cottonseed meal	115	93	89	75	257
Cottonseed burrs	322	195	84	101	380
Flaxseed	62	86	52	18	156
Kafir	19	39	45	38	122
Millet	41	160	93	97	350
Milo	22	56	45	32	133
Oats	111	60	103	79	332
Oat hulls	300	160	242	213	615
Peanut hulls	563	448	127	162	737
Wheat	26	39	58	40	137
Wheat shorts	56	73	89	62	225
Wheat bran	89	150	155	68	373
Rye	22	43	56	38	138
Timothy seed	33	46	41	34	121
Vetch seed	56	66	49	58	73
Soy beans	50	88	66	26	180
Cow peas	51	92	48	54	194
Field peas	48	178	51	50	279
Navy beans	50	55	60	64	180
Garden peas	37	94	47	61	202
Lima beans	36	21	66	42	129
Rice bran	128	92	70	41	203
Stringless beans	30	98	59	60	217
Serval	359	408	173	148	729

presented in table 3. The basic ration was a well-balanced cereal grain mixture fortified with protein, vitamins, and minerals. To this mixture had been added finely powdered wheat straw or peanut hulls as indicated in the table. An examination of the table indicates that the amount of indigestible residue is far greater than the crude fiber, and that there is no relation between the various fractions composing this matter.

A similar study was made of the digestibility of mixtures of feed consumed by sheep. Two rations were prepared, the

first of alfalfa, corn, and molasses, the second of alfalfa, oats, and molasses. Previous utilization studies had demonstrated that the oats ration was superior to that of corn (unpublished data). This same material, analyzed according to the new procedure, and presented in table 4, fails to indicate that the fiber fractions were responsible for the more favorable development.

TABLE 3

Apparent utilization of lignin, cellulose, and hemicellulose of mixed rat feeds

| | AMOUNT | MILLIGRAMS PER GRAM SAMPLE AND PERCENTAGE UTILIZATION | | | | |
		Crude fiber	Lignin	Hemi-cellulose	Cellulose	Total in-digestible residue
	gm.					
Basic ration	116	38	26	37	27	90
Feces	28	147	93	134	82	310
Coefficient of utilization		8%	13%	12%	26%	16%
Basic ration plus 15% peanut hulls	114	116	60	42	99	201
Feces	31	332	189	109	87	386
Coefficient of utilization		20%	13%	27%	75%	49%
Basic ration plus 30% peanut hulls	117	217	116	69	104	289
Feces	47	434	239	122	97	488
Coefficient of utilization		19%	18%	29%	62%	32%
Basic ration plus 20% wheat straw	90	106	55	62	111	228
Feces	25	290	171	169	94	435
Coefficient of utilization		24%	6%	23%	76%	45%
Basic ration plus 30% wheat straw	100	118	92	85	73	251
Feces	25	313	284	165	103	552
Coefficient of utilization		31%	9%	50%	73%	52%

At the completion of the experiment some of the animals were sacrificed, and samples from indicated levels of the stomach and intestines were similarly analyzed. The purpose of these tests was to ascertain if the ratio between the percentages of lignin, hemicellulose, and cellulose remained the same throughout the digestive tract. The results are tabulated in table 5, and the actual amount of each constituent is also

calculated in terms of its percentage of the remaining indigestible residue. An increase in the percent of lignin, for instance, in food passing through the intestine, indicates that it is being utilized less than one of the other two components.

TABLE 4

Utilization of cellulose, lignin, and hemicellulose of mixed feeds by sheep

SAMPLE	GRAMS	MILLIGRAMS PER GRAM SAMPLE				
		Fiber	Lignin	Hemi-cellulose	Cellulose	Total indigestible residue
Molasses	230	0	0	0	0	0
Alfalfa	454	255	180	112	153	445
Corn	230	19	23	49	45	117
Feces	239	328	183	128	163	476
Coefficient of utilization		34.7	49.3	40.8	50.7	50.1
Molasses	230	0	0	0	0	0
Alfalfa	454	255	180	112	153	445
Oats	230	143	60	103	79	332
Feces	245	382	318	140	152	610
Coefficient of utilization		36.9	18.4	42.9	57.4	46.0

TABLE 5

Lignin, cellulose, and hemicellulose of intestinal content of sheep

	MILLIGRAMS PER GRAM SAMPLE					PER CENT OF TOTAL RESIDUE		
	Crude fiber	Lignin	Hemi-cellulose	Cellulose	Total indigestible residue	Lignin	Hemi-cellulose	Cellulose
Corn ration								
Rumen	338	152	140	197	489	31	29	40
Reticulum	310	167	117	179	463	36	25	39
Abomasum	284	154	117	167	438	35	27	38
Omasum	330	160	108	184	452	35	24	41
Ileum	103	62	45	59	166	37	27	36
Ascending colon	301	170	123	161	454	37	27	36
Sigmoid colon	328	183	133	166	482	38	28	34
Oats ration								
Rumen	217	230	97	66	393	58	25	17
Reticulum	249	315	98	97	510	62	19	19
Abomasum	237	281	95	119	495	57	19	24
Omasum	218	332	78	89	499	66	16	18
Ileum	142	207	66	83	356	58	19	23
Ascending colon	371	305	138	161	605	50	23	27
Sigmoid colon	383	318	148	154	620	51	24	24

CONCLUSIONS

1. A modified method of determining lignin, hemicellulose, and cellulose of feeds is presented.

2. Both the total content of and the ratio between the quantities of lignin, hemicellulose, and cellulose of various cereal grains and grasses vary with the type of the material.

3. The indigestible residue determined by this method is much greater than the analogous crude fiber value.

4. The apparent utilization of cellulose in feeds is greater than that of lignin.

5. The cellulose of certain mixtures of feeds is better utilized than that of others; the utilization is somewhat determined by other ingredients of the mixture.

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THE INFLUENCE OF UREA INGESTION ON THE NITROGEN BALANCE AND ENERGY METABOLISM OF RATS [1]

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A survey of the literature dealing with the question of utilization of dietary urea by animals reveals that while some of the reports are conflicting (Krebs, '37), the preponderance of the more recent evidence is to the effect that urea can be at least partially utilized as a substitute for protein by polygastric animals, presumably by the aid of microorganisms. Positive results have been reported for sheep and cows (Honcamp and Koudela, '27; Schmidt et al., '37), for lambs (Sauer, '38), and for growing calves (Fingerling et al., '37; Bartlett and Cotton, '38; Hart et al., '38). The results reported for non-ruminants suggest the possibility of a species difference, but they are inconclusive.

Taylor and Ringer ('13) found that urea administered by mouth to dogs was eliminated quantitatively in the urine. In controlled feeding experiments with hogs Piepenbrock ('27) observed that the substitution of from 30 to 40% of the total protein of the ration by urea did not impair the gain in weight, thus indicating some utilization of urea by this species of animals. Moore et al. ('31) fed large quantities of urea to healthy human subjects, as part of their diet, and found that a substantial portion of the administered nitrogen was not recovered in the urine and feces. In these experiments the dietary

151

nitrogen was estimated from standard food tables. On the contrary, urea fed as the only source of protein in the diet to a human subject was found to be completely recovered as such in the urine by Kocher and Torbert ('32). There appears to be no record of experiments on this phase of the problem with rats.

A number of studies directed to the determination of the energy expenditure involved in the process of urea excretion are on record, and these are directly concerned with the effect of ingestion of urea on the total energy metabolism.

Using dogs as experimental subjects Tangl ('11) obtained only a slight increase in metabolism as a result of administration of urea by stomach. Lusk ('12) and Grafe ('15) found that the administration of urea to dogs had no effect on the heat production. In contrast to these observations, Lublin ('28) later reported that the administration of urea to dogs caused an increase in energy metabolism.

In experiments with human subjects, Borsook and Winegarden ('31 a, b, c) have observed a considerable increase in energy metabolism as a result of oral administration of urea. These results were questioned by Eaton, Cordill and Gouaux ('35) who have reported experimental data showing that ingestion of urea by human subjects had no effect on the heat production. Additional evidence against such an effect of urea in human beings is found in the recent work of Carpenter ('38).

Rajzman ('36) has found no rise in energy exchange after intraperitoneal injection of urea into rabbits and rats.

The purpose of the present investigation was twofold: first, in connection with an investigation of the general problem of specific dynamic effects of proteins and amino acids, it was desired to obtain, by the use of the same general technic, direct information on the controversial question of whether the excretion of urea exerts a specific dynamic effect; and, second, it was designed to show whether rats can utilize orally administered urea as a source of protein in the body.

EXPERIMENTAL PART

Six male albino rats weighing approximately 200 gm. each served as the experimental subjects. The rats were kept in individual cages and were subjected to the following dietary treatments during the periods and in the order indicated: (1) 8 gm. per day of a basal ration consisting of 93.7% of an approximately complete calf meal (Forbes, Kriss and Miller, '34) and 6.3% butterfat, for a period of 2 weeks, (2) 8 gm. of basal ration plus 2 gm. of urea per day, for a period of 8 days, (3) 8 gm. of basal ration for a period of 7 days, (4) 8 gm. of basal ration for 7 days followed by a fasting period of 31 hours. The food was offered in two equal portions, one at about 8 A.M. and the other at about 5 P.M.

The basal diet was of the following chemical composition: moisture, 10.3%; nitrogen, 3.25%; carbon, 42.26%; and energy, 4306 calories per gram. The analysis of the urea was as follows: moisture, 0.39%; nitrogen, 46.31%; carbon, 19.99%; and energy, 2514 calories per gram.

Urine and feces were collected from each rat during the last 7 days of the first period of feeding on the basal ration, and during the last 5 days on the urea supplemented diet. A small quantity of sodium fluoride was added to the urine as a preservative. This proved to be efficient, since determinations of nitrogen of urine preserved with sodium fluoride were found to agree well with those of urine collected in sulfuric acid from the same rats under identical dietary conditions.

The urine of each rat was analyzed for nitrogen and carbon. Energy was determined in a composite sample. The feces were first dried in an oven at approximately 60°C. and then exposed to the room air and allowed to come into equilibrium with the moisture of the air. After the weight of air-dried material for each rat was determined, the feces of all rats were combined and analyzed for nitrogen, carbon and energy.

At the end of each feeding period the rats were subjected to measurements of the respiratory exchange. These measurements began a few minutes after the rats had consumed their morning meal and continued for 7 consecutive hours. Finally,

the fasting metabolism was determined in a 7-hour period starting 24 hours after the last meal (of the basal ration) was fed to the animals. The measurements of the fasting metabolism entered into the computation of corrections of the total heat production for differences in body weight.

The apparatus used for measuring the respiratory exchange, the device for recording the movements of the animal in the respiration chamber, and the general technic employed were as described in a previous publication (Kriss, '38).

The protein metabolism was accounted for in the computation of the total metabolism in all feeding periods on the basis of the urinary nitrogen excretion in the basal period. The justification for this will become obvious from a consideration of the results of urinary analyses. The respiration and energy factors used in this computation were as determined by Kriss and Voris ('37) for beef muscle protein.

For comparative purposes the heat production was computed to a basis of uniform empty weight. The empty weights of the experimental subjects on the different dietary treatments were calculated from the total body weights on the basis of direct determinations of the alimentary fill of a separate group of rats of comparable size subjected to the same dietary treatments.

<div align="center">RESULTS</div>

The first indication that urea was not utilized by the rats to any significant extent for the building of body tissue was shown by the very slight changes in body weight resulting from the addition to the basal ration of 2 gm. of urea per day for a period of 8 days. A comparison of the weights of the rats at the end of this period with the weights attained at the end of the preceding basal period showed that the feeding of urea resulted in a slight loss in weight (from 1 to 7 gm.) in five of the six rats, and in a slight gain (2 gm.) in only one rat. Likewise, the omission of urea from the diet did not appear to affect the body weights to a significant extent.

More definite information regarding the question of utilization of urea is obtained from the chemical analysis of the urine (table 1) and from the balances of nitrogen, carbon and energy (table 2).

TABLE 1
Urinary analysis

| | RAT NO. | | | | | | AVERAGE |
	31	32	33	34	35	36	
Urine from basal ration							
Nitrogen per day, mg.	156	167	168	175	178	159	167
Carbon per day, mg.	139	140	135	142	143	130	138
C: N ratio	0.89	0.84	0.80	0.81	0.80	0.82	0.83
Energy per day, calories							1397
Urine from basal ration plus 2 gm. urea							
Nitrogen per day, mg.	1015	1078	1045	1065	1047	1056	1051
Carbon per day, mg.	516	541	526	532	538	539	532
C: N ratio	0.51	0.50	0.50	0.50	0.51	0.51	0.51
Energy per day, calories							6414
Differences due to urea							
Nitrogen, mg.	859	911	877	890	869	897	884
Carbon, mg.	377	401	391	390	395	409	394
C: N ratio	0.44	0.44	0.45	0.44	0.46	0.46	0.45
Energy, calories							5017
Energy: N ratio							5.68

TABLE 2
Utilization of dietary urea by rats

| CONSTITUENTS | DAILY INTAKE (2.0 GM. UREA) | LOST IN FECES | RECOVERED FROM URINE | | BODY BALANCES |
			Total	Per cent of intake	Per cent of intake
Nitrogen, mg.	926	7	884	95.5	+3.8
Carbon, mg.	400	3*	394	98.5	+0.8
Energy, calories	5028	38*	5017	99.8	—0.5

* Calculated from the fecal nitrogen. The determined value for carbon was 18 mg., and that for energy 208 calories.

The average C: N ratio of the urine obtained when the basal ration was fed was 0.83:1. The addition of 2 gm. of urea per day to the basal ration resulted in the lowering of the urinary C: N ratio to 0.51:1, the urinary residues from the ingested

urea having an apparent $C:N$ ratio of $0.45:1$. This ratio is almost identical with the $C:N$ ratio of urea $(0.43:1)$. The consistent character of the results of the individual animals is highly significant in this relation and these results indicate that the increased excretion of urinary nitrogen caused by the ingestion of urea was practically all in the form of urea. This is substantiated by the relation of energy to nitrogen found in the urine.

The effects of ingestion of urea on the daily balances of nitrogen, carbon and energy are summarized in table 2.

Of the total nitrogen ingested as urea 95.5% was recovered from the urine and 0.7% from the feces, leaving a positive balance of only 3.8%. It is unnecessary to assume that this small balance of nitrogen was in the form of protein.

Of the total intake of carbon and energy in the form of urea 98.5% and 99.8%, respectively, were found in the urine. If the small quantity of nitrogen lost in the feces be taken to represent urea, the carbon of the urine and feces could account for 99.2% of the carbon of urea fed, and the energy of the feces and urine would exceed the energy intake as urea by 0.5%. These small balances can be considered to be well within the limits of experimental error.

In a previous study (Kriss, '38), it was found that when different proteins (casein, gelatin and heart muscle) were fed to rats as supplements to a basal ration of the kind used in these experiments, considerable proportions of the nitrogen intake were retained in the bodies. When the proteins were fed in quantities of 1.5 gm. per day, the average percentages of their nitrogen retained were, for casein 6.0%, for gelatin 13.9%, and for heart muscle 29.1%. When these proteins were fed in quantities of 3.0 gm. per day the percentages of their nitrogen retained were, on the average, 17.2% for casein, 21.5% for gelatin, and 28.6% for heart muscle. In the light of these observations, and in consideration of the fact that the rats used in the present experiments were immature, as in the previous study, their inability to retain appreciable amounts of nitrogen from the ingested urea, as shown by the nitrogen

balances, may be taken as evidence of their nearly if not quite complete inability to utilize the urea nitrogen for the formation of protein.

It is not possible, of course, to determine from the balances of nitrogen whether an exchange took place between the nitrogen of the dietary urea and that of tissue proteins comparable to the exchange of nitrogen between dietary ammonia and tissue proteins as observed by Rittenberg and his co-workers ('39).

TABLE 3

Hourly heat production of rats per 200 gm. of empty body weight and total respiratory quotients as influenced by the addition of 2.0 gm. of urea to a basal maintenance ration

	RAT NO.						AVER- AGE
	31	32	33	34	35	36	
Basal ration, 8.0 gm. daily							
Initial period							
Heat production, calories per hour	913	929	925	870	886	979	917
Respiratory quotient	0.90	0.90	0.91	0.92	0.93	0.90	0.91
Final period							
Heat production, calories per hour	872	872	815	808	825	962	859
Respiratory quotient	0.91	0.92	0.95	0.95	0.93	0.93	0.93
Average of initial and final periods							
Heat production, calories per hour	893	901	870	839	856	971	888
Basal ration plus 2.0 gm. of urea							
Heat production, calories per hour	863	858	896	917	855	990	896
Respiratory quotient	0.90	0.91	0.90	0.92	0.93	0.91	0.91
Increase in heat production due to urea, calories per hour	— 30	— 43	+26	+78	— 1	+19	+8

Table 3 presents the data for the average hourly heat production of the individual rats computed per 200 gm. empty body weight, and the total respiratory quotients. The individual hourly measurements of CO_2 production, except those of the first hour of observation, are characterized as being very uniform; and of these, it was found necessary to omit from the averages only three single hourly determinations as being significantly affected by activity.

The effect of the feeding of urea on the heat production is shown by the comparison of the heat production representing

the urea-supplemented ration with the average heat production of the initial and final basal periods. Only one animal (rat 34) shows an appreciable increase in heat production as a result of the addition of urea to the basal ration. With the remaining five rats the differences in heat production attributable to urea are small, and they include some negative values. The average increase resulting from the feeding of urea is 8 gm.-calories per hour. This represents an increase of less than 1% of the heat production representing the basal ration and is not significant.

The respiratory quotients were not significantly altered by the addition of urea to the basal ration.

The foregoing observations lead to the conclusion that the feeding of urea, and its consequent excretion through the kidneys, did not appreciably affect the energy metabolism of the rats.

It appears that in rats, in contrast to ruminants and possibly swine, the synthesis of protein from urea does not occur in the intestinal tract to any significant extent.

SUMMARY AND CONCLUSIONS

Urea fed to rats as a supplement to a mixed maintenance ration, in quantities of 2 gm. per day, was almost entirely recovered as such in the urine and feces, as indicated by the balances of nitrogen, carbon and energy and by the ratios of carbon to nitrogen and energy to nitrogen in the urine. Of the total nitrogen ingested as urea 95.5% was recovered in the urine and 0.7% in the feces. Of the carbon and energy of the urea fed 98.5% and 99.8%, respectively, were found in the urine.

The feeding of urea showed no significant effects on either the heat production or the respiratory quotients.

The data lead to the conclusions that rats do not utilize urea to any significant extent, and that the excretion of urea does not exert a specific dynamic effect.

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PASTURE STUDIES XVI

THE NUTRITIVE VALUES OF KENTUCKY BLUE GRASS, RED TOP AND BROME GRASS

WITH PARTICULAR REFERENCE TO THE RELATION BETWEEN THE CHEMICAL COMPOSITION OF THE HERBAGES AND THE LIVE WEIGHT GAINS MADE BY THE ANIMALS SUBSISTING THEREON [1]

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Our present information from biological tests of the relative feeding values of the several species of grasses and clovers commonly occurring in mixed pasture herbage, is decidedly meager. It seems certain, however, that the herbage from different species of plants has an intrinsic nutritive value characteristic for each species (Knott, Hodgson and Ellington, '34; Kirsch, '33; Pollard and Chibnall, '34; Garrigus and Rusk, '35; Crampton, '34; Crampton and Cameron, '36).

Tests at Macdonald College (Crampton and Forshaw, '39) with rabbits have also indicated a definite intra-seasonal trend in the feeding value of mixed pasture herbage. Clippings of herbage grown during spring and fall supported normal growth of rabbits, while material grown during midsummer under less favorable climatic conditions proved of

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decidedly poor value. In these studies interpretation of the results was complicated by the change in botanical composition of the herbage with advancing season. It has been shown (Dore, '36) that, in the permanent pastures of Quebec province, wide variations may exist between the proportions of the various species in a pasture as a result of management and of climatic conditions. To eliminate this factor it became apparent that clippings from pure stands of the grasses to be studied would be necessary and in 1938 such material was made available.

Furthermore, it was becoming increasingly evident that differences in nutritive value of pasturage as measured by the live weight gains of rabbits were frequently not predictable from the usual chemical feeding stuffs analysis (Crampton, '39). This situation, if applicable also to the nutritive value of such materials for larger animals, is disturbing in view of the large amount of pasture research already completed in which chemical analyses alone are the criterion of 'nutritive value.'

The literature contains strong evidence (Crampton, '39) that the usefulness of the partition of the carbohydrates into crude fiber and nitrogen-free extract for the purpose of describing nutritive value is to be seriously questioned. Norman ('39) states, "It would be an important advance if the determination of the crude fibre fraction and its use in expressing composition were abandoned as inadequate, unreliable, and misleading." He further states, "The most serious criticism of the present system of analyses used for grasses and forage crops is that there is included no measure of the lignin content."

It seemed desirable, therefore, in the 1938 feeding trials to design the experiment in such a way as to permit a critical statistical examination of the relation between the chemical composition (proximate principles of the feeding stuffs analysis) of the clippings fed and the growth of the animals subsisting thereon.

EXPERIMENTAL PART

Diets

From large plots ($\frac{1}{4}$ to $\frac{1}{2}$ acres) of pure stands of brome, Kentucky blue and red top grass clippings were made at about 16-day intervals throughout the summer of 1938. These clippings were dried in a forced draught hot air dryer and subsequently ground in a hammer mill to pass a $^{15}\!/_{32}$ inch screen. In order to obtain sufficient herbage for the feeding periods it was necessary with all three grasses to combine the clippings of July 11th and August 4th and those of August 29th and September 19th. Thus at the end of the growing season five samples of herbage clippings were available from each of the three grasses above mentioned. The chemical analyses of these fifteen lots of herbage are given in table 1.

Animals

Sixty young growing rabbits, averaging 1500 gm., were allotted equally to fifteen groups, each group to be fed on a different clipping.

All animals were penned and fed individually. The feeding practice and equipment have been previously described (Crampton, '34). The trials were of 35 days duration. The first 7 days on feed were treated as a preliminary period while the last 28 days constituted the test period proper. Throughout the trial feed was provided ad libitum. Weekly live weight gain and feed intake data were recorded for each animal.

STATISTICAL ANALYSIS OF DATA

The method of partial regression was employed as suggested by Crampton and Hopkins ('34) to adjust the observed live weight gains of the rabbits in order to remove the effects on gain of varying levels of feed intake and of different initial weights of rabbits. The initial weights shown in table 2 are those at the beginning of the test periods, and are those involved in the adjustment of gains above referred to.

In an attempt to evaluate the usefulness of chemical analyses as an index of nutritive value of pasture herbage simple, partial and multiple correlations were calculated between the average 28-day gains of the rabbits of each lot and the feed fractions isolated (1) by the standard scheme of feeding

TABLE 1

Chemical analysis of dry matter of clippings used

DATE	SPECIES	NITRO-GEN[1]	ETHER EX-TRACT[1]	ASH[1]	CRUDE FIBER[1]	N-FREE EXTRACT[2]	LIGNIN[3]	CELLU-LOSE[4]	OTHER CH₂O[5]
	Brome	4.3	2.6	15.9	15.6	39.3	6.8	19.2	28.9
May 26	Kentucky blue	4.0	2.5	11.5	21.7	39.5	5.3	20.6	35.3
	Red top	3.6	3.1	11.8	18.7	40.1	5.8	19.2	33.8
	Brome	4.0	3.2	16.9	19.1	35.6	7.3	20.9	26.5
June 10	Kentucky blue	3.3	2.7	11.8	22.4	42.8	7.3	23.0	34.9
	Red top	3.1	2.7	11.2	23.7	59.4	7.2	24.4	51.5
	Brome	3.4	4.6	14.0	23.0	37.3	7.8	22.0	31.1
June 23	Kentucky blue	3.7	3.9	12.0	23.9	37.3	8.0	25.5	27.7
	Red top	3.3	3.9	11.8	26.8	36.9	8.7	26.8	28.2
July 11 }	Brome	3.5	4.0	11.9	21.0	41.0	6.8	22.6	32.6
Aug. 4 }	Kentucky blue	2.6	3.5	10.4	23.5	46.2	9.3	22.8	37.6
	Red top	3.1	3.4	9.9	24.7	42.4	10.5	25.0	31.6
Aug. 29 }	Brome	3.8	2.8	13.5	17.9	42.4	7.6	19.1	33.6
Sept. 19 }	Kentucky blue	4.1	2.7	12.2	18.7	41.0	9.8	19.1	30.8
	Red top	3.8	2.6	13.1	18.0	42.7	9.1	18.7	32.9

[1] Official A. O. A. C. methods.

[2] N-free extract = D.M.—((nitrogen × 6.25) + ether extract + total ash + crude fiber).

[3] Determined by method proposed by Crampton and Maynard ('38) with the modification that acid hardened filter paper replaced bolting silk in the recovery of the pepsin digestion residue and that the boiling of the chloroform-acetic acid suspension of lignin was discontinued immediately the surface scum broke, and that diatamaceous earth in place of asbestos was used in the final lignin filtration.

[4] Determined by method proposed by Crampton and Maynard ('38).

[5] Other carbohydrates = D.M. —((Nitrogen × 6.25) + ether extract + totol ash + lignin + cellulose).

stuffs analysis and (2) by the scheme of analysis proposed by Crampton and Maynard ('38). In addition standard and partial regression coefficients [4] were determined using in each instance the 28-day gains as the dependent, and the feed fractions (as protein, fiber, etc.) as independent variables.

[4] Snedecor—Statistical Methods, 1937, Chapter 13.

TABLE 2

Initial weights of rabbits, dry matter consumption and live weight gain data

DATE	SPECIES	INITIAL WEIGHT	DRY MATTER CONSUMPTION	28-DAY GAINS	28-DAY GAINS ADJUSTED FOR DEVIATIONS FROM THE GENERAL MEANS OF INITIAL WEIGHT AND DRY MATTER CONSUMPTION
		gm.	*gm.*	*gm.*	
	Brome	1911	2905	261.25	280
May 26	Kentucky blue	1889	3150	253	269
	Red top	2006	3397	274.5	187
	Average	1935	3067	262.92	
	Brome	1505	2524	85.75	128
June 10	Kentucky blue	1586	2910	170.5	131
	Red top	1553	3059	221.75	139
	Average	1548	2831	159.33	
	Brome	1383	2635	109.75	103
June 23	Kentucky blue	1300	2692	38.75	3
	Red top	1335	2507	—110.25	—94
	Average	1339	2612	12.75	
July 11 ⎫ Aug. 4 ⎭	Brome	1398	2473	147.5	184
	Kentucky blue	1365	2353	—149.0	—89
	Red top	1384	2741	104	71
	Average	1382	2522	34.17	
Aug. 29 ⎫ Sept. 19 ⎭	Brome	1382	2621	259.75	256
	Kentucky blue	1361	2314	83.25	152
	Red top	1314	2428	178.75	211
	Average	1352	2454	173.92	
Lot averages	Brome	1516	2631	172.8	190
	Kentucky blue	1500	2634	79.3	93
	Red top	1518	2826	. 133.75	103
General average		1511	2697	128.62	129

RESULTS AND DISCUSSION

The 28-day gains, adjusted for differences in initial weight and feed intake, are given by clippings and by species of grass in table 3.

A perusal of these gains shows that in addition to intra-seasonal variations in growth promoting ability there are significant differences between species. Also there appear to

be differences in the part of the season at which these grasses display signs of declining nutritive value. While all three species show a mid-season falling off in nutritive value the 'low' is not as marked with brome grass as in the case of either Kentucky blue or red top.

Particularly poor growth was obtained with those clippings of Kentucky blue grass and red top taken during the mid-summer. In fact two rabbits on the Kentucky blue grass and one on the red top of July 11th and August 4th clipping

TABLE 3

Twenty-eight-day gains of rabbits adjusted for differences in initial weight and feed intake

DATE OF CLIPPING	BROME GRASS	KENTUCKY BLUE	RED TOP	CLIPPING MEAN
	gm.	gm.	gm.	gm.
May 26	280	269	187	245
June 10	128	131	135	132
June 23	103	3	—94	4
July 11 } Aug. 4 }	184	—89	71	55
Aug. 19 } Sept. 19 }	256	152	211	207
Species average	190	93	103	129

Necessary difference (with P = 0.05) between (1) means of clippings of same species = 98 gm.; (2) means of species with same date of clipping = 98 gm.
Necessary difference between clipping means = 56 gm.
Necessary difference between species means = 44 gm.

died before the completion of the 35-day feeding period. The data for these rabbits were deleted and three replacements used to complete the data for the test. While these replacements succeeded in living through the feeding period their growth response paralleled those of the others of the group.

At the completion of the 35-day period, one of the replacement rabbits on Kentucky blue grass and one of the original animals that survived the first 28-day test feeding period were continued, the latter (no. 1) for 28 days and the former (no. 2)

for 9 days on the same diet with the addition of 10 gm. daily of cornstarch. The results are shown below.

RABBIT	AVERAGE DAILY GAIN ON KENTUCKY BLUE GRASS GROWN BETWEEN JUNE 23RD AND AUGUST 4TH	AVERAGE DAILY GAIN ON SAME GRASS PLUS 10 GM. DAILY OF CORNSTARCH
	gm.	gm.
1	—4.5	+9.09
2	+1.0	+4.4

The results secured by supplementing the diets with cornstarch and the condition of the animals at the completion of the trial would indicate that lack of available energy was at least one of the factors responsible for the decline in growth noted with mid-summer herbage. This is in agreement with the findings of Crampton and Finlayson ('35) and Crampton and Cameron ('36) using casein and cane sugar as supplements respectively.

The results of the statistical studies are summarized in table 4.

Simple and partial correlations

Before proceeding further, attention is called to the magnitude of the correlations needed for statistical significance shown in table 4 a. The fact that they are large numerically is due to the small number of degrees of freedom available for the usual tests of significance. It may be pointed out that significance is probability. Thus when a correlation may not in nineteen out of twenty cases prove real (i.e., significant with odds of 19:1 or $P = 0.05$) it may prove real in eighteen cases out of twenty (i.e., $P = 0.10$), etc. Thus the correlation obtained is the best estimate of the facts from the data. Our dependence on them is determined by what arbitrary limit of odds we demand, which after all is in part a matter of judgment of the case.

In this study it was necessary in calculating the correlations to treat the average gains of the four rabbits on each diet as single observations. Thus the quantities correlated were not individual observations but means of four, which

will be correspondingly less subject to the irregular fluctuations obscuring true correlations. The reduction in the significance of the correlations obtained is therefore probably

TABLE 4

Correlations between 28-day gains of rabbits and fractions isolated by feeding stuffs analyses

PROXIMATE PRINCIPLE	SIMPLE CORRE-LATION COEF-FICIENTS (r)	PARTIAL CORRE-LATION	STANDARD REGRESSION COEFFICIENTS		PARTIAL REGRES-SION COEF-FICIENTS (b)	MULTIPLE CORRE-LATIONS
			Actual	Relative %		
Standard analysis						
Crude protein	+0.6582	+0.7274	+0.6933	35.6	+28.61	
Ether extract	—0.7815	—0.5944	—0.4394	22.6	—75.33	$R^2 = 0.863$
Ash	+0.1864	—0.6546	—0.4272	22.0	—35.63	
Crude fiber	—0.7436	—0.2002	—0.1383	7.1	— 5.32	$R = 0.929$
N-free extract	+0.1619	+0.4774	+0.2468	12.7	+ 5.06	
Modified scheme [1]						
Crude protein	+0.6582	+0.6950	+0.5757	29.7	+23.73	
Ether extract	—0.7815	—0.5556	—0.3281	16.9	—56.40	$R^2 = 0.905$
Ash	+0.1864	—0.6864	—0.3944	20.4	—32.89	
Lignin	—0.4866	—0.5413	—0.1857	9.6	—14.42	$R = 0.952$
Cellulose	—0.7396	—0.5501	—0.2935	15.3	—12.58	
Other carbohydate	+0.2266	+0.3014	+0.1596	8.2	+ 3.09	

[1] Crampton and Maynard ('38).

TABLE 4 a

Correlation coefficients necessary for statistical significance $(P = 0.05)$

	DEGREES OF FREEDOM	STANDARD ANALYSIS	MODIFIED ANALYSIS
Simple correlations	13	0.532	0.532
Partial correlations			
Standard analysis	7	0.666	
Modified scheme	6		0.707
Multiple correlations	7 and 6	0.860	
	6 and 7		0.900

more apparent than real. The interpretation of these statistics in the discussion following has been made with the peculiarities and limitations of these data in mind.

Simple correlations in data of this type must be interpreted in the sense of cause and effect with extreme caution, for obviously there is not likely to be a direct causal relationship between any of the feed fractions and the live weight gain of the rabbits. For example, the crude fiber correlation of —0.7436 doubtless results from the common association of crude protein with both gain and fiber.

Some of the difficulty from common associations may be eliminated by using partial rather than simple correlations. When this method is applied it is seen that much of the apparent relation of crude fiber to gains has been eliminated; that of crude protein and of nitrogen-free extract has been increased. Ash also becomes of importance. This is in accord with the diluent effect to be expected of any inorganic substance present to the extent of 10 to 17% of the total dry matter of the diet (see table 1).

From the squared multiple correlation coefficient (R^2) it is seen that in the case of the standard analysis scheme 86.3% of the variation in gains is accounted for by the five feed fractions isolated; and in the case of the modified plan, 90.5% is the result of the six fractions considered.

From the standard regression coefficients one may obtain an idea of the relative importance of any one of the proximate principles in causing variability in gain. Thus of the 86.3% of variability in gain accounted for by the five fractions of the standard feed analysis, some 35.6% is traceable to variations in protein content, 22.6% to ether extract, etc.

It appears from these figures that crude fiber and nitrogen-free extract have probably played minor roles in this connection as compared to protein, fat and ash. In the modified plan of analysis, all values are reduced due to the inclusion of six instead of five fractions. Nevertheless, in this latter plan, the total weight of the three carbohydrate fractions is 33.1% of the variability accounted for by the analysis as compared to 19.8% in the standard analysis; from which it might be argued that some improvement in the chemical

description of the growth promoting properties of the feed has been effected by the modified plan.

Partial regression coefficients indicate the change in gain (grams live weight in 28 days) to be expected with a change of one unit of per cent in the feed fraction in question.

The effect of the fractions other than those of the carbohydrate group may at first appear large. It will be noted, however, that changes in chemical composition in these fractions are not large in relation to the marked differences in the gains obtained on these several clippings. Furthermore it is by no means probable, for example, that an increase of 1% of ether extract is nutritionally merely an increase in a high energy utilizable food material.

Digestion studies during 1938 at Macdonald College (unpublished) on diets of pasture herbage both with steers and with rabbits show ether extract to be variable in digestibility. For example, in pasture herbage grown during mid-summer the ether extract content is greater than that found either in spring or fall grown herbage. The digestibility of this ether extract fraction of the summer herbage is, however, markedly lower. The increase in ether extract is probably due to the presence of seeds in the mature summer herbage. These seeds, however, largely escape digestion, thus increasing the ether extract of the feces. In green herbage the fact that ether removes such substances as chlorophyl, phytosterol and phosphatides, as well as the triglycerides and free fatty acids, also contributes to the variable utilization by animals which this feed fraction may have. Furthermore an increase in ether extract may be accompanied by qualitative changes in other fractions which result in lowered feeding value. Thus, change in the ether extract content may be an index of change in feeding value much greater than a simple addition effect of the ether extract itself. This is doubtless in part the situation with ash though here the diluent effect is probably the greater. It is likely that the rather high ash values indicate the incidental but unavoidable inclusion of some soil with the lawn mower clippings.

Protein being highly digested was related to gains in a positive way. In view of the results with cornstarch already mentioned, it seemed improbable that this correlation meant that even the lowest protein content (16.25%) represented suboptimum protein intake. Rather, due to the unavailability of carbohydrate and fats, the protein was providing an important source of energy; and increases in protein meant increases in available dietary energy and hence increases in live weight gains.

Admittedly the problem is by no means solved. It is evident that the present scheme of feeding stuffs analysis is quite inadequate for describing the growth promoting properties of the energy yielding nutrients of pasture herbage. Hence, the assumption that samples of pasture herbage are of different feeding value because of differences in chemical composition cannot be accepted as warranted.

SUMMARY AND CONCLUSIONS

Evidence is presented that in addition to differences in the nutritive value of different species of grasses commonly found in the pastures of Eastern Canada, the feeding value of the herbage of any single species changes during the growing season. Herbage grown during spring and fall, when plant growing conditions are favorable, is of excellent feeding value. On the contrary, herbage grown during mid-summer is of lower nutritive value, due apparently to a reduction in the availability to the animal of the carbohydrate fractions.

Statistical examination of the data indicates that simple correlations between growth of the animals and the fractions of the usual feeding stuffs analysis may frequently point to erroneous conclusions when interpreted in the sense of cause and effect. Partial correlation changes not only the magnitude but also the sign of some of the coefficients. In particular it may be noted that variations in crude fiber, which under simple correlations appear important, probably actually have only minor effects on live weight gains. Also ether extract and ash are highly negatively correlated with gain.

Protein content is highly correlated with gain in weight of animal. However, there is no evidence that protein as such is a limiting factor in the growth promoting value of immature pasture herbage, but rather that increases in protein probably represent merely increases in available energy.

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THE RECTAL TEMPERATURE AND THE METABO-
LISM OF THE WILD COTTONTAIL RABBIT

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To contribute information as to whether there are metabolic differences between animals in the wild and the domestic state, measurements were made on eight wild cottontail rabbits (Lepus sylvaticus). These were snared in Oklahoma on November 29, 1937, and were either mature or nearly so. Measurements of rectal temperature and metabolism were made by the methods previously used for domestic rabbits (Lee, '39 a, b).

Rectal temperature. As there is a temperature gradient in the rectum of the wild rabbit, a condition also found in the domestic rabbit, the thermo-junction should be inserted 150 mm. to obtain valid body temperature measurements. The gradient between the temperatures at 75- and 150-mm. depths averaged 0.3°C. at an air temperature of 28°C. and was slightly less at lower air temperatures. Fasting enforced for 24 hours did not alter the rectal temperature. Between environmental temperatures of 16° and 28° the body temperature remained constant, but when the rabbits had been 24 hours at 10° to 12°C., it was lowered 0.7°C., on the average. Three to 4 minutes of activity caused increases in the temperature of 0.3° to 2.1°C., the maximum being greater than that noted with our domestic rabbits after similar exercise and approaching that of 2.5° reported by Purdy ('25). At environmental temperatures of 15° to 28°C., when the rabbits had food available or when fasting had been enforced up

to 24 hours, the range in rectal temperatures (eighty-four measurements) at a depth of 150 mm. was from 38.7° to 40.9° C. The average was 39.9° C., nearly the same as that of our domestic rabbits (39.6°). There was no sex difference.

Prerequisites for basal metabolism measurements. The wild rabbits were quiet, as a rule, but any periods of activity were rejected and only the values obtained in quiet periods and agreeing within 10% of the minimum measurement on each day were used in calculating the average metabolism. The zone of thermic neutrality was found to be between 28° and 30° C. Measurements at 28° and then at 30° C. on the same day gave the following results, expressed as calories per square meter of surface area ($S = \frac{K \times w^{2/3}}{10,000}$ or $S = 0.001 w^{2/3}$) per 24 hours:

Rabbit no.	11	12	13	15	16	19	Average
28° C.	561	631	713	611	659	717	649
30° C.	539	671	700	598	661	712	647

These measurements were made after the rabbits (fasting 24 hours) had been living previously at a moderate temperature and during the 24 hours just beforehand at 28° C. Observations above 30° and below 28° gave higher values. A series at 28° immediately after the rabbits had been removed from an environment of 12° C. gave results from 6 to 29% above subsequent measurements after 24 hours at 28° C. After 22 to 30 hours of enforced fasting at 28° to 30° C. (exact length of fasting unknown, because of uncertainty as to when the rabbits ate last), the respiratory quotients ranged from 0.69 to 0.82 and averaged 0.73. The heat production reached a fairly uniform level after 24 hours of fasting at thermic neutrality and did not decrease when the fast was continued for several hours longer. The prerequisites for basal metabolism measurements on the wild rabbit are, therefore, absence of activity, thermic neutrality, 24 hours of habituation to thermic neutrality, and fasting for 24 hours at this temperature.

Basal metabolism. The basal metabolism of each of the rabbits was determined at intervals over 8 months (table 1). With five of the eight rabbits the heat production per square meter

of body surface was highest in the initial experiment. For rabbits 19, 20, and 22 the environmental temperature previous to their initial experiments was only 18°C., which was undoubtedly a contributory cause of their first extraordinarily high measurements. In the second series of experiments, about 3 weeks

TABLE 1

Basal metabolism of the wild rabbit

Rabbit no. and sex	11 ♀	12 ♂	13 ♂	15 ♂	16 ♂	19 ♀	20 ♀	22 ♀
Average weight, kg.	0.97	0.90	0.96	0.87	0.90	1.01	1.02	1.22
APPROXIMATE DATE	CALORIES PER SQUARE METER [1] PER 24 HOURS							
December 5 [2]	628	799	780	700	753	816 [3]	906 [3]	791 [3]
December 27	649	704	804	751	659	726	667	722
February 15	573	666	730	729	653		624	644
February 25						672		615
March 2	606							584
March 15	622			672			657	576
March 20	570	656		764		713	648	526
March 25		726	634	648	619	699	624	571
April 4	561	631	713	611	659	717	592	558
May 10	557	665	732	671	643	602	531	573
July 15		626	710	649				
Average [4]	591	668	721	687	647	688	620	597
	TOTAL HEAT PRODUCTION PER 24 HOURS							
Average	59.2	62.7	70.8	62.3	61.5	70.4	63.7	68.8

Measured at 28° to 30°C., after 22 to 30 hours of enforced fasting; habituated to experimental temperature for 24 hours prior to experiment.

[1] Weight in grams raised to the two-thirds power and multiplied by 0.001.

[2] First experiment.

[3] Rabbits 19, 20 and 22 were not at 28° to 30° prior to first experiment but at 18°C.

[4] Excluding first experiment.

later, most of the rabbits had a higher metabolism than in succeeding measurements. Probably prior to their arrival at the laboratory (December 2, 1937) they had been living at much lower temperatures than usually prevailed at the laboratory, and adjustment to moderate temperatures had not fully taken place even at the time of the second measurements. Any decrease in metabolism as the animals' stay in the laboratory be-

came longer may have been the result of habituation to a higher and narrower range of environmental temperatures rather than of adaptation to handling. As the metabolism is predisposed to be high in the first experiment with any animal, the first experiment has been omitted from the general average in each case.

The measurements on the individual animals were fairly uniform. The maximum variation from the individual averages was +21% with rabbit 22 and ±14% or less with the others. The standard deviation of the percentage differences of the daily average values from the general average for each rabbit was ±6.7%, comparable to that of ±6.9% noted with our domestic rabbits. The average heat production of the individual wild rabbits ranged from 591 to 721 calories per square meter, and the general average for the eight rabbits was 652 calories.

The average total 24-hour heat production ranged from 59 to 71 calories and averaged 64.9 calories (average weight, 0.98 kg.). This is 6% above the average total heat production of our domestic rabbits of like weight (Lee, '39c) and recalls the finding of Benedict and Petřík ('30) that wild rats have a higher metabolism than laboratory-bred albino rats.

There was no consistent relationship between variations in rectal temperature and variations in basal metabolism.

The heat production of the four females averaged 8% below that of the four males. This sex difference is greater than the 2% difference found with our domestic rabbits. It is questionable, however, whether this finding (8%) based on so few values is any more than suggestive of the trend.

Reaction of metabolism to environmental temperature. The fasting heat production (calories per square meter) of three wild rabbits measured at 16° after at least 24 hours' habituation to this temperature and their average basal metabolism at 28°C. were as follows:

Temp.	No. 19	No. 20	No. 22
16°C.	930	1011	901
28°C.	688	620	597

At 16°, with no indication of shivering shown by the activity records, the heat production was increased, on the average, 49% above the average basal metabolism at 28°C.

SUMMARY

The average rectal temperature of eight wild cottontail rabbits, essentially adult and having an average weight of 0.98 kg., was 39.9°C. at a depth of 150 mm. in the rectum. The temperature increased as the depth in the rectum at which the measurement was made increased. Between the 75- and 150-mm. depths, the average difference in temperature was 0.3°C. (environmental temperature, 28°). Fasting enforced for 24 hours, at air temperatures between 10° and 28°, did not alter the body temperature. Environmental temperatures between 16° and 28° had no effect, but at 10° to 12°C. the average rectal temperature was lowered 0.7°. Three to 4 minutes of exercise caused increases in temperature of 0.3° to 2.1°C.

The average total heat production per 24 hours was 64.9 calories, and the average heat production per square meter of surface area ($S = 0.001w^{2/3}$), 652 calories per 24 hours. The basal metabolism of the wild rabbit is 6% higher than that of the domestic rabbit of the same average weight. At 16°C., the metabolism was 49% above the basal level.

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THE INFLUENCE OF VARIETY, SEASON AND GREEN MANURES UPON THE COMPOSITION OF WHEATS

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A wide variation has been found to occur in the ash, calcium, magnesium, potassium, iron, phosphorus and sulfur content of wheat collected from different parts of Utah (Greaves and Hirst, '29). This may be due in part to the variation in composition of the soil, yet the soils thus far examined contain large quantities of the essential mineral plant elements. Hence, other factors, such as, for example, the quantity of irrigation water applied during the growing season which has been shown to change materially the composition of wheat, oats, barley and corn (Greaves and Nelson, '25; Greaves and Carter, '23; Greaves and Hirst, '29), may play a predominating role. The present data are the results of a study of the influence of variety, season and manurial treatments upon the ash, calcium, magnesium, potassium, iron, sulfur and phosphorus content of wheats.

The wheats under consideration were grown on the Nephi dry-land substation located about 5 miles south of Nephi, Utah, on the north slope of the Levan Ridge. The soil is of the deep dry-farm type derived from the weathering of the adjacent mountain ranges and contains phosphates, potassium and large quantities of gypsum. The soil of the farm is a clay loam, and contains in the surface foot-section as an average 4100 pounds of nitrogen, 7000 pounds of phosphorus, 86,000 pounds of potassium, 1480 pounds of sulfur, 42,000 pounds of organic carbon, 69,000 pounds of magnesium carbonate, and 139,000 pounds of calcium carbonate. Consequently,

it is rich in these plant food elements. Water is the limiting factor of crop production, the average annual precipitation being 13 inches.

The results reported in this paper are from analyses of wheats grown on this soil. The samples were obtained as follows: Each yearly sample was composed of three replications of individual varieties. The samples were analyzed yearly for 8 years according to the methods of the Association of Official Agricultural Chemists ('24) for ash, calcium, magnesium, phosphorus and sulfur. The analyses for potassium cover a period of 3 years, and those of iron 5 years.

The results for ash, calcium, magnesium, iron and phosphorus for seven spring wheats are given in table 1. They are the averages of two determinations on each variety for each of 8 years. Variance was calculated by using the yearly percentages as replicates.

Although the differences between the varieties of wheat approach the point of significance, they cannot be judged significant on the basis of variation of the same wheat from year to year.

The results give a picture of the ash and ash constituents of these seven varieties of spring wheat when grown on a typical dry-farm soil.

The average composition of these spring dry-farm wheats is as follows: Ash, 1.58%; calcium, 0.071%; magnesium, 0.192%; potassium, 0.391%; iron 0.0058%; phosphorus, 0.298%; and sulfur, 0.213%.

The mineral composition of seventeen varieties of winter wheat, grown on the same soils and under similar conditions except that the seeding was done in the fall, is given in table 1.

A significant difference in these wheats was found in the calcium content, but the differences with respect to the other constituents are not significant, though they approach the 5% point. The average composition of these winter wheats follows: ash, 1.40%; calcium, 0.068%; magnesium, 0.157%;

potassium, 0.361%; iron 0.0064%; phosphorus, 0.276%; and sulfur, 0.188%.

The averages for the winter wheats are lower than those for the spring wheats. These results are of special importance in that we here have a measure of the comparison of spring and winter wheats. Approximately one-twentieth

TABLE 1

Average percentages of ash, calcium, magnesium, potassium, iron, phosphorus and sulfur in varieties of wheats

	ASH	Ca	Mg	K	Fe[1]	P	S
	%	%	%	%	%	%	%
Spring varieties							
Regenerated defiance	1.65	0.038	0.194	0.423	0.0056	0.296	0.207
Kota	1.60	0.081	0.205	0.364	0.0060	0.327	0.243
Marquis	1.60	0.076	0.177	0.387	0.0062	0.291	0.194
Kubanka	1.58	0.080	0.170	0.387	0.0058	0.315	0.204
Early baart	1.58	0.072	0.239	0.438	0.0054	0.287	0.211
Chul	1.54	0.072	0.163	0.368	0.0059	0.284	0.203
Hard federation	1.52	0.075	0.196	0.360	0.0060	0.288	0.231
Average	1.58	0.071	0.192	0.391	0.0058	0.298	0.213
Winter varieties							
Utac	1.71	0.085	0.178	0.408	0.0068	0.307	0.223
Kofod	1.53	0.060	0.169	0.421	0.0058	0.302	0.188
Regal	1.42	0.074	0.169	0.332	0.0070	0.289	0.170
Tenmarq	1.29	0.062	0.162	0.312	0.0055	0.284	0.177
Montana no. 36	1.35	0.070	0.163	0.356	0.0058	0.282	0.179
Sevier no. 34	1.46	0.068	0.158	0.406	0.0061	0.280	0.194
Kanred	1.45	0.068	0.178	0.361	0.0066	0.279	0.197
Kharmont	1.34	0.067	0.153	0.355	0.0068	0.277	0.198
Kharkof	1.36	0.065	0.169	0.347	0.0062	0.276	0.180
Turkey and Kofod	1.34	0.062	0.158	0.373	0.0060	0.273	0.197
Sevier no. 59	1.43	0.066	0.133	0.366	0.0068	0.272	0.195
Alton	1.33	0.072	0.165	0.366	0.0056	0.271	0.170
Black hull	1.40	0.074	0.063	0.333	0.0062	0.268	0.188
Kharkof Hayes no. 2	1.30	0.070	0.162	0.376	0.0064	0.268	0.174
Turkey no. 926	1.32	0.059	0.160	0.362	0.0064	0.264	0.206
Turkey	1.36	0.072	0.170	0.353	0.0088	0.258	0.189
Newturk	1.42	0.068	0.153	0.304	0.0063	0.247	0.164
Average	1.40	0.068	0.157	0.361	0.0064	0.276	0.188

[1] All the samples of wheat were ground in an iron mill; consequently they would receive iron from this source. See E. B. Forbes, F. M. Bugle and J. E. Mensching ('13).

of the ash of these wheats is calcium and about one-ninth magnesium, or there is approximately twice as much magnesium as calcium.

These averages for ash, calcium and magnesium are slightly lower than those reported for wheats of the state of Utah (Greaves and Hirst, '29). This is due to the fact that the wheats here reported are all dry-farm wheats. The earlier data contained analyses of both dry-farm and irrigated wheats. Nevertheless the present results are higher than those given by Lawes and Gilbert (1884), Konig (1893) and Sherman ('37). They are higher in calcium and magnesium and lower in potassium, phosphorus and sulfur than Ohio wheats (Forbes, Bugle and Mensching, '13).

The calcium-phosphorus ratio of these wheats is approximately 1 to 4, thus indicating that a supplement high in calcium should be added when the wheats are fed. Alfalfa samples thus far analyzed show a calcium-phosphorus ratio of 6 to 1; hence it is highly probable that any legumes raised on highly calcareous soil make excellent supplements for these wheats.

The question naturally arises as to whether the differences which have been found in the mineral content of spring and winter wheats would manifest themselves if the wheats were fed to animals. This has been answered by Greaves and Greaves ('33) for Kota, a high calcium and phosphorus spring wheat, and Turkey, a low calcium and phosphorus winter wheat. These wheats were fed to young albino rats 22 to 25 days old for 3 weeks. The animals were kept in individual cages on a diet consisting of 90% wheat and 10% of the following mixture: 24 gm. casein, 32 gm. yeast, 12 gm. sodium chloride and 17 gm. cabbage. Fifteen replications were used in the case of the Kota and ten in the case of Turkey. The animals fed Kota made better gains and had better bones as shown by the higher percentages of ash, calcium and phosphorus. Moreover, the serum calcium and phosphorus in all animals fed Kota were higher than in those fed Turkey.

In order to learn if there is a relationship between the various ash constituents, correlations were made according to the formula given by Wallace and Snedecor ('31); they are presented in table 2.

The wheats were ground in an iron mill and only 1-year analyses were made of the iron. This may account for the absence of a positive correlation between iron and the ash and other ash constituents. However, there is a highly significant correlation between most of the other mineral constituents. Most of the sulfur (Greaves and Bracken, '37) and phosphorus of these wheats is in organic combinations; hence, the close relationship of these elements is shown in a

TABLE 2

Correlations between the various constituents of twenty-four varieties of wheat. These figures are based upon the average quantities of material found in the wheat from 1923 to 1935

	Fe	P	Ca	K	S	Mg
.P	—0.325 [1]					
Ca	0.007	0.551 [1]				
K	—0.233	0.479 [1]	0.281 [2]	0.449 [1]		
S	—0.104	0.628 [1]	0.459 [1]	0.449 [1]		
Mg	—0.203	0.492 [1]	0.459 [1]	0.525 [1]	0.607 [1]	
Ash	—0.218	0.718 [1]	0.721 [1]	0.626 [1]	0.651 [1]	0.602 [1]

[1] Highly significant.
[2] Significant.

highly significant correlation between the calcium of these wheats and the phosphorus, sulfur, magnesium and ash, but no significant correlation is observed between the calcium and potassium, and iron.

A significant correlation is indicated between the potassium of these wheats and the calcium, and a highly significant correlation between the potassium and the phosphorus, sulfur, magnesium and ash.

The sulfur content also varies directly with the quantity of the other constituents with the exception of iron. These wheats are especially high with respect to phosphorus and ash. The correlations in the cases of magnesium and ash and the

other constituents with the exception of iron are also highly significant. Hence it may be concluded that insofar as these dry-farm wheats are concerned, and tentatively insofar as the dry-farm wheats of the state are concerned, a high ash content indicates a high phosphorus, calcium, potassium, sulfur and magnesium content.

The twenty-six varieties were analyzed separately during each of 8 years, giving a good comparison for years. The average results obtained each year are given in table 3.

TABLE 3

Average percentages of ash, calcium, magnesium, potassium, iron, phosphorus, and sulfur in twenty-four varieties of wheat during years 1923 and 1929–1935

CONSTITUENT	1923	1929	1930	1931	1932	1933	1934	1935
	%	%	%	%	%	%	%	%
Ash	1.580	1.450	1.390	1.610	1.520	1.440	1.090	1.550
Calcium	0.083	0.069	0.075	0.066	0.068	0.065	0.070	0.069
Magnesium	0.199	0.173	0.181	0.171	0.202	0.169	0.142	0.150
Phosphorus	0.285	0.213	0.305	0.345	0.298	0.286	0.230	0.328
Sulfur	0.190	0.225	0.206	0.190	0.174	0.178	0.164	0.182
Potassium	0.389	0.359	0.385					
Iron	0.004	0.007	0.006	0.006	0.006			

A large variation is shown in the ash from year to year. In 1934 it was 1.09%, whereas in 1923 it was 1.58%, a difference of 45%. Calcium shows only slight variation, whereas magnesium varied 40%. The phosphorus and sulfur in these wheats both show a high yearly variation. The iron content shows very little, if any, variation. This may be due to the fact that the wheats were milled in an iron mill.

Influence of green manures

During 8 years, Utah Kanred winter wheat was grown on soil which received treatments as indicated in table 4.

The data, averages of which are shown in table 4, were analyzed by variance and, as indicated, a highly significant difference was found for wheat yields as influenced by the various treatments. Similarly, a highly significant difference was found for percentage of calcium contained in the wheat.

For phosphorus the difference was only significant with odds of 20:1 that the variation was not due to chance alone. The percentages of ash, magnesium, and sulfur showed no definite trend.

It is interesting to note that the addition of green manures to these soils increased the phosphorus content of the wheat. This, in all probability, resulted from the fact that the soil

TABLE 4

Average acre yields and average percentage composition with respect to ash, calcium, magnesium, phosphorus and sulfur of Utah Kanred wheat grown on soil receiving different manurial treatments

	AVERAGE YIELD PER ACRE	AVERAGE COMPOSITION				
		Ash	Ca	Mg	P	S
	bushels	%	%	%	%	%
Fall plowed for fallow	19.19	1.91	0.067	0.176	0.299	0.19
Peas plowed when in pod	16.52	1.71	0.068	0.180	0.302	0.18
Peas plowed when in bloom	15.35	1.62	0.070	0.175	0.313	0.18
Peas plowed when 12 inches high	16.19	1.53	0.072	0.184	0.317	0.19
Peas plowed when 6 inches high	17.92	1.60	0.068	0.188	0.291	0.19
Wheat plowed when in milk	12.23	1.70	0.071	0.193	0.314	0.16
Wheat plowed when in bloom	13.17	1.67	0.072	0.174	0.334	0.17
Wheat plowed when 12 inches high	14.90	1.59	0.069	0.182	0.331	0.17
Wheat plowed when 6 inches high	17.22	1.56	0.067	0.180	0.317	0.17
Fall plowed for fallow	18.45	1.52	0.063	0.179	0.287	0.19
All straw plowed under	18.92	1.53	0.062	0.171	0.299	0.18
All straw burned	19.65	1.58	0.065	0.191	0.278	0.18
High headed straw plowed	19.43	1.61	0.066	0.187	0.305	0.17
High headed straw burned	20.08	1.52	0.065	0.179	0.273	0.17
Significant differences, Odds 20:1		1.85	...	0.0089	....	0.031	...
Highly significant difference, Odds 100:1		2.44	...	0.0112	....	....	...

carries an abundance of total phosphorus, but the quantity available during the growing season is limited by the weather, cultural treatment and the quantity of organic material decomposed. Inasmuch as the soil is low in organic matter the quantity decomposed each year is small, but when fresh organic material is added bacterial activity is increased with a resulting increase in the available phosphorus, which is reflected in the phosphorus content of the grain.

CONCLUSIONS

Spring wheat grown on the Nephi substation dry farm carries greater percentages of ash, calcium, magnesium, potassium, iron, phosphorus and sulfur than does winter wheat grown on the same soil. A significant difference was found in the calcium content of different varieties of winter wheat grown on the same soil. A highly significant correlation was found to exist among most of the mineral constituents of wheat. When the wheats of different years were compared, a high variation was found to occur in the various mineral constituents from year to year. The addition of green manures to a typical dry farm soil caused a highly significant variation in the ash content and a significant difference with respect to calcium and phosphorus. The use of green manures materially increased the phosphorus content of wheat probably due to its effect in increasing bacterial activities which in turn increased the available plant food in the soil.

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IRON METABOLISM

1. THE ROLE OF CALCIUM IN IRON ASSIMILATION [1]

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A conviction, apparently widely shared by those engaged in nutritional research, attributes to calcium the faculty of increasing iron absorption from the intestinal tract or in otherwise aiding in its economy. It is based, as nearly as the author can determine, on the initial work of von Wendt ('05) and that of Sherman ('07). The study to be reported here yielded contrary findings.

The observation that an anemia developed in a high-tumor strain of mice fed a diet consisting wholly of a commercial dog biscuit led to this work. An analysis of this diet revealed the presence of an unusually high percentage of calcium, amounting to more than $3\frac{1}{2}\%$. Since this product constituted the sole article of diet for mice in these laboratories over a period of years, the question arose as to what, if any, the relationship of the calcium to the observed anemia might be, and whether or not this relationship contributed to a larger syndrome which terminated in the production of new growths.

That an iron-calcium antagonism was involved in the production of the anemia in our mice was further suggested by certain results of Rose and Vahlteich ('32). They found that when adequate and similar amounts of iron and copper in the form of whole wheat grain or as the ash of the same supplemented with milk were fed to anemic rats, lower hemoglobin

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readings were obtained when the iron and copper were administered in the form of the ash than in the form of the whole grain. No explanation was offered for their observations. It occurred to us that these observed differences might be explained on the basis of the greater amount of milk consumed and the correspondingly greater calcium intake in the case of the animals receiving their iron and copper as part of the ash of the whole wheat.

Both von Wendt ('05) and Sherman ('07) determined iron retention in human adult subjects by means of balance experiments. Their experimental periods were a matter of days. Because of the shortcomings inherent in an experimental procedure of this nature, we decided to use rats and to determine the total iron retention as revealed by chemical analysis of the entire animal after a period of 5 to 6 weeks of controlled feeding. A simple basal diet was used and the only variable was the addition of various supplements of calcium salts.

EXPERIMENTAL PART

Basal diet 1 had the following percentage composition: ground yellow corn (ball-milled) 42.5; wheat flour [2] 42.5; commercial casein [3] 9; dried yeast [4] 3; cod liver oil 2; sodium chloride, C.P., 1. Ferric citrate U.S.P.[5] was added to make the iron content equal to 50 mg. per kilo. Rations were weighed out daily and were equalized, insuring an average 10 gm. daily intake for the duration of the feeding period. This diet provided a daily intake of 0.5 mg. of iron and 0.02 mg. of copper per animal.

In an attempt to determine the effect of calcium supplementation on a whole wheat ration, the following diet was used: ground whole wheat (ball-milled) 90%; commercial casein 5; dried yeast 2; cod liver oil 2; and sodium chloride, C.P., 1%. The calcium supplements were introduced as sub-

[2] Patent flour (Pillsbury XXXX).
[3] Crude casein from the Casein Mfg. Co. of America, Bainbridge, N. Y.
[4] Powdered yeast foam tablets from the Northwestern Yeast Co., Chicago, Ill.
[5] U. S. P. VIII-Scales (Merck) ball-milled.

stitutes for equivalent amounts of wheat. A 10 gm. daily intake of the diet furnished 0.5 mg. of iron and 0.07 mg. of copper.

The rats were of the Wisconsin and the large Yale albino strains. At 20 to 22 days they were placed on a dried whole milk [6] diet. When their hemoglobin values had reached a level of 4 gm. or less per 100 cc. of blood, which required from 2 to 3 weeks, representative animals were sacrificed for anemic controls and the remaining rats were distributed equally for the feeding of the basal and experimental diets.

All animals were subjected to exsanguination before their tissues were separated and prepared for analysis. This was effected by perfusion with Locke's solution under ether anaesthesia, using a technic which was adapted from the methods of Whipple ('26) and Bethke, Steenbock and Nelson ('23). The blood and perfusate were collected on specially prepared sponges of hospital gauze which had been prepared by extraction with dilute HCl and subsequent washing with distilled water. Analyses showed either that they were free of iron or that only negligible amounts were present. The degree of success attained in our exsanguination procedure was determined by expressing the total blood iron values in terms of grams of hemoglobin, and with the aid of blood volume data from Donaldson's monograph ('15) stating the hemoglobin in terms of grams per 100 cc. of blood. These values were in turn compared with those for hemoglobin determined by the Newcomer method, using a standardized Bausch and Lomb filter, the blood for this purpose having been obtained from the tail immediately before the sacrifice of the animal. Using such a criterion as a means of comparison, it appears that we were able to remove 90% and upward of all blood. Essential to a good perfusion are light anaesthesia, continued respiratory movements, and the introduction of from 25 to 40 cc. of saline solution, the latter depending upon the size of the animal. The significance of the removal of the blood from the solid tissues is evident when it is recalled that

[6] Klim.

blood constitutes only 6 to 8% of the weight of the animal, yet it contains from 55 to 65% of the total body iron. Following exsanguination, the liver and the spleen were removed, sliced, washed in physiological saline, weighed, and dried on watch glasses in an electric oven at 100°C. and then stored in glass containers until analyzed. The intestinal tract was removed, freed of all residues, washed, and added to the carcass, which was then dried in a manner similar to the liver and the spleen. The sponges were placed on watch glasses and dried over radiators with the necessary precautions.

Iron determinations were made according to a modified Elvehjem procedure ('30) for substances high in phosphorus. All ignitions were effected in quartz evaporating dishes by means of Meeker burners or in an electric muffle furnace. Prolonged heating on a boiling water bath and on electric hot plates according to Eggleton and Eggleton ('29) without the addition of alkali effected hydrolysis of pyrophosphates. Color fading was not experienced under our operating procedures and quantitative recovery of added iron was the rule.

RESULTS

The results of our initial experiment involving the addition of 1% of $CaCO_3$ to the basal diet are given in table 1. The carcass iron includes that of all organs except those specifically mentioned in the table.

An analysis of the results reveals that the addition of 1% of $CaCO_3$ has reduced the iron content of all tissues except the spleen, the average liver, blood, and carcass values being 57, 86 and 90% of the respective control values. The livers of the calcium-supplemented animals average less than the livers of the anemic controls sacrificed at the beginning of the experimental feeding.

In table 1 are also recorded the results of feeding 3% of $CaCO_3$. The accentuated effect of increased calcium supplementation is evidenced by the fact that the average liver, blood, and carcass values are only 28, 72 and 61% of the respective control values.

TABLE 1

The effect on total iron retention by rats of the addition of calcium carbonate to the basal diet over a period of 5 weeks

DIET	ANIMAL		IRON CONTENT			
	No.	Body weight	Liver	Spleen	Blood	Carcass
		gm.	*mg.*	*mg.*	*mg.*	*mg.*
Basal	141	150	0.640	0.110	3.658	1.870
only	143	168	1.010	0.138	3.740	2.620
	145	175	0.624	0.093	4.064	2.245
	147	226	0.535	0.117	4.675	2.730
	149	196	0.762	0.107	4.275	2.300
Average		183	0.714	0.107	4.168	2.286
Basal	142	155	0.356	0.096	3.280	1.950
plus	144	170	0.374	0.094	3.517	1.845
1% of	146	205	0.444	0.111	3.794	2.245
$CaCO_3$	148	203	0.454	0.119	3.781	2.175
Average		183	0.407	0.105	3.593	2.053
Anemic controls (4)		104	0.459	0.083	0.862	0.785
Basal	441	163	1.130		3.662	2.595
only	443	155	0.412	Included	3.594	2.550
	445	200	0.472	with	4.347	3.310
	447	185	0.560	carcass	3.328	2.570
	449	165	0.485		3.507	2.355
Average		173	0.611		3.687	2.676
Basal	442	173	0.218		2.968	1.865
plus	444	178	0.153		2.798	1.820
3% of	446	156	0.194		2.085	1.720
$CaCO_3$	448	175	0.151		3.052	1.645
	450	146	0.171		2.654	1.405
	451	160	0.166		2.315	1.345
Average		164	0.175		2.645	1.633
Anemic controls (4)		104	0.134		0.533	0.438
Basal	4413	200	0.310	Included	4.241	2.730
only	4415	201	0.362	with	4.108	2.525
	4417	161	0.546	carcass	3.231	2.100
	4419	177	0.770		3.837	2.210
Average		186	0.497		3.604	2.391
Basal plus	4414	141	0.139		1.523	1.266
calcium lactate	4416	148	0.123		1.966	1.190
equivalent to	4418	108	0.115		1.090	0.896
2½% of $CaCO_3$	4420	145	0.116		1.454	0.976
Average		135	0.123		1.508	1.082
Anemic controls (5)		76	0.166		0.774	0.607

The results of feeding calcium lactate in amounts equivalent to 2½% of $CaCO_3$ are given in table 1. These indicate that the depressive effect of calcium is increased when calcium is supplied in this form. In this case the average liver, blood and carcass values are only 25, 42 and 45% of the respective control values.

The results of feeding $CaCl_2$, $Ca_3(PO_4)_2$, and $CaSO_4$ in amounts equivalent in calcium content to 3% of $CaCO_3$

TABLE 2

The effect on total iron retention by rats of the addition of calcium chloride, tri-calcium phosphate, and calcium sulphate equivalent in calcium content to 3% of calcium carbonate over a period of 5 weeks

DIET	ANIMAL		IRON CONTENT			
	Litter no.	Body weight	Liver	Spleen	Blood	Carcass
		gm.	*mg.*	*mg.*	*mg.*	*mg.*
Basal	1	163	1.000	0.082	3.774	2.064
only	2	178	1.227	0.120	3.424	2.056
	3	165	0.578	0.112	3.390	1.992
	4	187	1.110	0.072	3.520	2.880
Average		173	0.979	0.096	3.527	2.243
Basal	1	82	0.206	0.089	1.892	1.104
plus	2	57	0.127	0.093	1.318	1.010
$CaCl_2$	3	57	0.093	0.101	1.285	0.796
	4	135	0.110	0.103	2.041	1.220
Average		83	0.134	0.096	1.659	1.032
Basal	1	126	0.163	0.095	2.221	1.292
plus	2	155	0.220	0.122	3.200	1.824
3% of	3	159	0.222	0.148	3.365	1.724
$CaCO_3$	4	165	0.288	0.077	3.422	2.236
Average		151	0.223	0.110	3.052	1.769
Basal	1	141	0.148	0.092	2.755	1.580
plus	2	184	0.396	0.214	4.117	1.940
$Ca_3(PO_4)_2$	3	148	0.479	0.145	3.372	1.600
	4	170	0.199	0.070	3.077	2.200
Average		161	0.305	0.130	3.330	1.830
Basal	1	130	0.769	0.071	4.562	1.856
plus	2	162	0.610	0.120	3.397	2.000
$CaSO_4$	3	146	0.962	0.301	3.471	2.012
	4	148	0.806	0.082	3.565	2.176
Average		147	0.787	0.143	3.749	2.011
Anemic controls (7)		66	0.360	0.081	1.262	0.706

(table 2) show that these other calcium salts with the exception of CaSO₄ have a similar adverse effect on iron retention. The unpalatability of the CaCl₂ diet reduced consumption by 25%. The remaining animals were maintained on a 10 gm. daily intake.

TABLE 3

The effect on total iron retention by rats of the addition of calcium carbonate to a whole wheat diet over a period of 60 days

DIET	HEMOGLOBIN PER 100 CC.		ANIMAL		IRON CONTENT			
	Initial	Final	Body weight	Litter no.	Liver	Spleen	Blood	Carcass
	gm.	*gm.*	*gm.*		*mg.*	*mg.*	*mg.*	*mg.*
Wheat	2.03	12.51	165	1	0.707	0.200	4.528	3.795
diet	2.65	12.05	165	2	1.117	0.290	4.315	2.890
only	3.05	14.20	145	3	0.884	0.159	4.174	2.275
	3.05	14.90	185	4	0.612	0.159	4.520	3.125
	2.72	12.77	164	5	0.946	0.196	4.434	2.680
Average	2.70	13.28	165		0.853	0.201	4.394	2.953
Wheat	2.29	12.77	161	1	0.738	0.257	4.525	1.845
diet	2.71	10.71	165	2	0.724	0.266	3.715	2.295
plus	3.47	11.29	158	3	0.589	0.140	4.207	2.330
1% of	3.59	12.05	173	4	0.887	0.284	4.515	2.325
CaCO₃	3.06	13.47	159	5	0.634	0.186	4.252	2.184
Average	3.02	12.06	164		0.714	0.227	4.241	2.196
Wheat	2.29	13.47	155	1	0.692	0.135	4.431	2.845
diet	2.89	11.59	143	2	0.521	0.094	3.692	1.885
plus	3.87	12.05	165	3	0.579	0.107	4.305	2.295
3% of	4.89	13.19	171	4	0.798	0.156	4.611	2.655
CaCO₃	3.85	12.77	180	5	0.380	0.148	4.564	2.080
Average	3.56	12.61	163		0.594	0.128	4.321	2.352
Anemic controls (5)		4.17	97		0.586	0.059	0.883	0.960

In table 3 are recorded the results of calcium supplementation of a modified whole wheat diet over a period of 60 days. Starting with the lowest initial hemoglobin readings, the unsupplemented-wheat animals show the highest average hemoglobin regeneration and the highest average iron values for carcass, blood and liver. The calcium-supplemented animals retained only 82.5% of the iron of the unsupplemented-wheat animals. The differences between the 1 and 3% CaCO₃ groups are less obvious; blood regeneration was equally good;

while the iron values for liver and spleen of the 1% $CaCO_3$ group were superior to those of the 3% $CaCO_3$ group, the reverse was true for the blood and carcass values.

The data as analyzed thus far, with the exception of $CaSO_4$, point to the conclusion that under the conditions which have been described, calcium exerts an adverse effect on iron assimilation. Kletzien ('35) previously had held that $CaSO_4$, too, acts to diminish iron assimilation; later and more extensive observations do not substantiate this. The depressive effect is likewise evident in the case of the citrate, acetate, tartrate, and malate of calcium, the data of which will be incorporated in a paper bearing on the effect of anions on the iron assimilatory process. Evidence has also been accumulated indicating that calcium oxalate acts like the sulphate. In these experiments, with the exceptions noted, the initial milk-induced anemia was not corrected, or only partially so, by diets supplemented with calcium, in spite of an adequate iron and copper intake. It seems reasonable to regard the anemia in our mice as due to the high calcium content of the commercial dog biscuit, which constituted their sole dietary. The results of Rose and Vahlteich ('32) could be explained on a similar basis.

DISCUSSION

An analysis of our tissue iron values seems to indicate that in the animal economy, the synthesis of hemoglobin predominates over that of other iron-containing respiratory pigments, suggesting the possibility of a deficiency of the latter, together with disturbed oxygen relationships, even though the blood picture is not obviously that of anemia.

From the comparative studies of Macallum ('26) on the mineral constituents of tissue fluids and sea water, and the studies of Johnston and Ball ('30), Ravdin et al. ('32), and de Beer et al. ('35) on the mineral composition of the secretions of the alimentary tract, it is suggested that in the present investigation we are dealing with a disturbance in the normal concentration of the common inorganic ions in the principal

absorptive portions of the digestive tract. That the intro-
duction of readily ionizable or metabolizable salts of calcium
within the intestinal tract may provoke an ionic imbalance
with concomitant disturbances within or at the protoplasmic
boundaries does not seem to be mere idle speculation. This
may serve to explain the results in table 2 where the use of
$CaCl_2$ gave effects different from those yielded by $CaSO_4$. The
corrosive effects of $CaCl_2$ are probably not due to its com-
position as such, but are related rather to its ready solubility
and high degree of ionization, overcoming thereby the ionic
antagonism of the Na and K and interfering with the normal
reactions involving chlorides, carbonates, and phosphates.
That adsorption is a factor in making iron unavailable is not
disproved; neither are changes in intestinal pH or the removal
of iron by precipitation.

Gardner and Burget ('38) observed that KCl added to
glucose solutions increased the absorption of glucose from
chronic closed intestinal loops in dogs; the contrary was
observed when $CaCl_2$ was introduced in similar concentra-
tions. These results confirmed in certain respects earlier
work of Magee and Sen ('32) and Gellhorn and Skupa ('33).
Robscheit- Robbins and her co-workers ('28) pointed to "salts
as perhaps responsible" for the marked superiority of apricots
for hemoglobin regeneration. Insofar as the iron and copper
required for hemoglobin regeneration are concerned, this
explanation is very plausible, in view of the marked pre-
ponderance of K and Na in apricots in contradistinction to
their content of Ca and Mg.

That the retention of copper is affected by calcium sup-
plementation in a manner similar to that of iron is sug-
gested by other unpublished data of ours. The association
of deficiencies of other minor inorganic elements such as
iodine, manganese, cobalt, zinc and boron with excessive
calcium supplementation is suggested. Reference to iodine in
this connection was made by Kletzien ('34) in an explanation
of results of Levine, Remington and v. Kolnitz ('33). The
identification of perosis (slip tendon disease of fowl) with

manganese deficiency by Wilgus et al. ('36) and the known high incidence of this disease with use of diets heavily supplemented with calcium may also be cited as evidence for such an association. The occurrence of certain "salt lick" diseases of animals subsisting on vegetation grown on soils of a calcareous origin and their correction by fertilizing with traces of these minor elements offers additional corroborative evidence. Similarly, the identification of so-called "goitre belts" with regions having a history of limestone origin may be cited.

SUMMARY

1. Young rats were fed a milk diet productive of a severe anemia, following which the effect of variation in the supply of calcium on the amount of iron in the various organs and the entire body was studied.

2. The addition of 1 and 3% of calcium carbonate to the basal diet resulted in lower tissue iron values. This was also true when calcium in the form of the lactate equivalent to $2\frac{1}{2}$% and the chloride and tri-phosphate equivalent to 3% of calcium carbonate were fed. This was not true, however, in the case of calcium sulphate.

3. The addition of 1 and 3% of calcium carbonate to a basal diet containing 90% of ground whole wheat resulted in lower tissue iron values.

4. The possible bearing of these findings on the metabolism of other minor elements is discussed.

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THE PROTEIN CONTENT OF THE ORGANS AND TISSUES AT DIFFERENT LEVELS OF PROTEIN CONSUMPTION [1]

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TWO FIGURES

The effect of variation in protein intake on the total quantity of protein in the body has been estimated from the nitrogen content of the body by Forbes, Voris, Bratzler and Wainio ('38) and by Hamilton ('39). However, the observations of Addis, Poo and Lew ('36) as to the individual manner in which different organs respond to fasting and protein refeeding make it quite likely that these total changes are only the sum of a series of quite dissimilar reactions. If that be the case, it becomes a matter of some practical importance as well as of general interest to determine whether different levels of protein intake are associated with differences in the distribution of total protein within the body (organ protein per 100 gm. total protein).

From a colony that had been reared on a diet containing 18% of protein, 120 rats about 90 days of age were selected. They were divided into six groups of twenty rats each so that the average body weight of each group was 150 gm. One of these groups was immediately sacrificed and the protein content of the organs and tissues determined, using the methods described by Addis, Poo, Lew and Yuen ('36). The other five groups were given diets that contained 6, 11, 16, 27 and 43%

199

of protein, respectively. These diets were almost exactly iso-caloric since the protein variation was obtained by substituting casein for cornstarch. An air-dry commercial brand of casein was used; it contained 79.2% of protein. Each diet consisted of 60% by weight of a casein cornstarch mixture with the concentration of casein ranging from 4% at the lowest level through 10, 16 and 30 to 50% for the diet richest in protein. In addition each diet contained in per cent lard 15, sardine oil 10, dry yeast 9, air-dry ground alfalfa 2, and Osborne and Mendel's salt mixture 4%. After 18 days on these diets the protein content of the organs and tissues of each of the five groups was determined and compared with the amounts found in the group in which the measurements were made at 0 days. The results are given in table 1.

Since, at the start, each of the six groups had the same average body weight and represented samples chosen at random from the same population, we can obtain the quantities of protein newly formed in growth over a period of 18 days on these five levels of protein intake by subtracting the amounts found at 0 days from those found after 18 days. In order to facilitate relative comparison, the changes in total protein and in the protein content of the kidney, liver and blood serum have been expressed as percentages of the original quantities found at the start and plotted in figure 1 against the quantities of food protein in the 18-day period.

The examples given in figure 1 are sufficient to show that when protein consumption rises from amounts not even sufficient for maintenance through quantities adequate for growth to luxus levels there is a diversity in the effect produced on the various organs of the body. Thus, the kidney protein continues to rise with every increase in protein consumption but the maximum addition of protein to the liver is attained on the 27% protein diet, while with serum protein the greatest content is reached on 16% protein ration. A consideration of the results given in table 1 will show that this is also true for the other organs and tissues, so that the effect on the total body protein, which reaches its maximum at the mid-point in

TABLE 1

Fresh weight and protein content per rat of organs and tissues before and after 18 days of increasing protein consumption

No.	Protein content	Protein eaten in 18 days	ORGAN¹ AND TISSUE EXAMINATION MADE	Kidney	Heart	Liver	Alimentary tract, etc.	Clot	Serum	Uterus	Carcass	Total
	%	gm.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
S²	18	...	Weight	0.995	0.519	6.567	25.701	2.452	2.876	0.290	110.6	150.0
			Protein	0.161	0.083	1.256	1.631	0.712	0.166	0.040	19.497	23.546
1	6	9.5	Weight	0.853	0.545	5.435	22.750	2.150	2.341	0.223	108.6	142.9
			Protein	0.142	0.086	0.959	1.510	0.610	0.139	0.032	20.397	23.874
2	11	17.8	Weight	0.950	0.585	6.656	24.147	2.454	2.850	0.358	122.7	160.7
			Protein	0.158	0.097	1.229	1.658	0.692	0.186	0.049	22.619	26.687
3	16	27.1	Weight	1.092	0.622	7.398	28.083	2.884	3.215	0.358	133.3	177.0
			Protein	0.173	0.105	1.406	1.705	0.838	0.215	0.049	22.963	27.455
4	27	43.8	Weight	1.165	0.619	7.367	27.200	2.841	3.003	0.389	135.4	178.0
			Protein	0.189	0.100	1.454	1.746	0.819	0.199	0.055	23.603	28.166
5	43	80.0	Weight	1.191	0.534	6.595	26.400	2.571	2.661	0.355	125.1	165.4
			Protein	0.195	0.091	1.353	1.762	0.781	0.180	0.051	22.770	27.184

¹ Definitions of these organs and tissues as used in this study are as follows: 'Total Protein' includes all the protein of the rat, even that of the hair, but not the protein of the food within the alimentary tract. 'Kidney' is both kidneys stripped of their capsules, split open with a razor blade and blotted on filter paper for removal of blood and urine. 'Heart' is the organ removed in a uniform manner by section of the large vessels close to the muscle, and after removal of blood from the interior of the auricles and ventricles. 'Liver' is the organ after blotting on filter paper to remove surface blood. 'Alimentary Tract, etc.,' includes the gastro-intestinal canal from the diaphragm to close to the anus, the pancreas, spleen, adrenals, ovaries, mesentery, and bladder, and includes all the fat of the abdominal and pelvic cavities. After boiling in 0.5 molar sodium acetate solution at pH 5 the gastrointestinal tract is opened with scissors and the contents removed by washing under running water. 'Clot' and 'Serum' were separated by centrifuging at high speed for 30 minutes all the blood that could be obtained after cutting the abdominal aorta of the anesthetized rat while the heart was still beating. In a healthy rat about 75% of the total circulating blood volume is thus obtained. 'Uterus' includes the uterine horns stripped of fat and cut at the entrance into the vagina. Fluid contents are removed before weighing. 'Carcass' is the whole rat after exsanguination and removal of the heart and all abdominal and pelvic organs. This remainder consists predominantly of the muscle, skin and skeleton although the protein of the lungs, nervous system and peripheral vascular system is also included. 'Total Weight' is the live body weight and includes the contents of the gastrointestinal tract. 'Alimentary Tract etc. Weight' includes the weight of the gastrointestinal contents.

The protein quantities in the group killed before the diets were started were published in a previous paper (Poo, Lew and Addis, '39). It should be noted that in the fresh weights for this group given in that paper there is an error due to transposing part of the carcass weight to the weight of the alimentary tract, etc.

² Stock diet on which the animals subsisted before being placed on experiment.

the range of protein consumption, is only the sum of a series of quite individual organ and tissue reactions.

The concentration of protein in the organs and tissues (protein per 100 gm., fresh weight) can be obtained from the protein quantities and organ weights given in table 1. These concentrations vary in magnitude from 6.1% for the alimentary tract, etc. (where the protein was diluted by the abdominal pelvic fat), to 30.8% for blood clot. In the carcass and alimentary tract, etc., the protein concentrations seem to vary inversely with the amount of visible fat, for they are lowest on the 16% protein diet, where fat deposition is at a maximum,
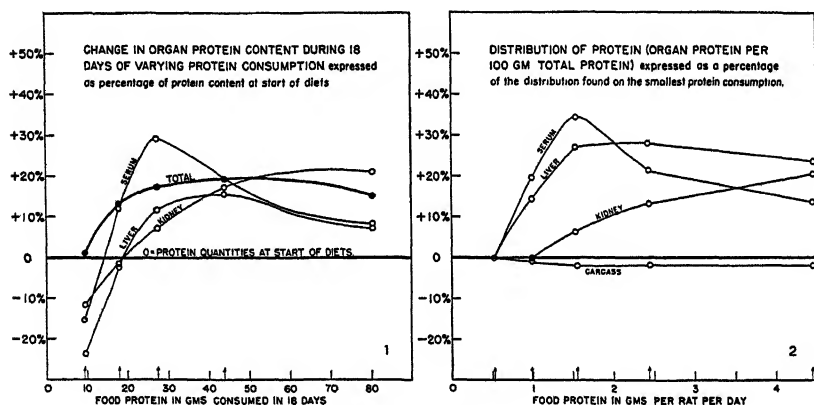
Figures 1 and 2

and rise progressively in the leaner groups that were given either too little or too much protein.

In the serum and blood clot, and in the heart, kidney and uterus, there is no regular change in concentration with change in protein consumption. On the other hand, in the liver, where glycogen as well as fat and water is an appreciable part of the total weight, the protein concentrations change with each increment of food protein, increasing from 17.7% on the 6% ration to 20.5% on the 43% protein diet.

The distribution of organ protein (organ protein per 100 gm. total protein) is a measure that is independent of varia-

tion in the fat and water content or in the total body weight of the groups we are comparing. Under the conditions we have chosen, we pass from a supply of food protein so restricted that protein catabolism exceeds anabolism, through two gradations of adequate protein consumption to an amount so excessive that the rate of anabolism again becomes diminished. Under these diverse circumstances, each organ and tissue is in competition with all the others for its share of the total available protein, and alterations in the proportion that each in the end obtains, may be taken as an index to its relative functional importance in the total economy, if it is accepted that the protein content of an organ is the best available measure of its total operative metabolic machinery. These distributions can be deduced from the protein quantities given in table 1. The relative change in the proportions of the total protein allocated to various organs and tissues is shown in figure 2, where the distribution found on the lowest protein diet is taken as 100% and the distributions found at successively higher levels of protein consumption are expressed as a percentage of these quantities.

Figure 2 shows that as the protein intake increases, the proportions allocated to such internal organs as the kidney and liver, and to such tissues as the serum, increase while there is a slight fall in the proportion assigned to the carcass, the part that contains more than 80% of the total protein. But it is not justifiable to generalize from these facts to the statement that the external and peripheral musculature, bone and skin lose and the internal organs gain, for there is no regular or definite change in the alimentary tract, etc., or heart as the protein intake increases. For certain organs at least, what happens may be better understood by considering how the change in conditions may influence not only the economy as a whole, but especially how it may affect the function of each organ in particular. In the case of the kidney we know that every increase in protein consumption above the quantity needed for maintenance and growth requires an increase in the osmotic work of the kidney, and it is reasonable to ascribe

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the steady rise from the level on the 11% protein diet in the proportion of the total protein assigned to the kidney to the hypertrophy that follows increased work (Walter and Addis, '39). Although we cannot see clearly how increase in protein consumption may influence the many functions of the liver, it is at least a matter of interest that the hypothesis that serves for the kidney is shown by figure 2 to be inadequate for the liver, since this organ does not increase its share of total protein with luxus protein consumption but declines as the total anabolism diminishes. There are other organs and tissues (e.g., the uterine changes secondary in all probability to an endocrine effect) in which the structural alterations may not be related to change in function. But in every organ and tissue the effect of increasing protein consumption is an individual one so that it is impossible to predict the effect on any part of the body from the change in the protein of the body as a whole.

SUMMARY

Diets containing 6, 11, 16, 27 and 43% of protein, respectively, that were almost isocaloric and that contained adequate and equal mineral and vitamin concentrations were constructed by substituting casein for cornstarch. These diets were given for 18 days to five groups of rats, twenty rats in each group, each group having at 0 days an identical body weight of 150 gm. On the eighteenth day the protein content of various organs and tissues was determined. A sixth group of rats similar in every respect to the others was killed at 0 days and the change that had occurred in the diet groups was determined by subtracting the quantities found at 0 days from the protein quantities found on the eighteenth day.

The greatest total gain occurred on the diet containing 27% of protein but each organ and tissue had its own mode of reaction to an increasing supply of food protein. Some organs made their maximum gain of protein on the 16%, some on the 27% and others on the 43% protein diet.

Each level of protein intake was associated with its own characteristic pattern of distribution of protein (organ protein per 100 gm. total protein).

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IRON AND COPPER VERSUS LIVER IN TREATMENT OF HEMORRHAGIC ANEMIA IN DOGS ON MILK DIETS [1]

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The widespread tendency among clinicians to adduce superior though unexplained properties to liver for treatment of hemorrhagic anemias brought the question to us of whether liver would actually prove superior to iron and copper in treatment of dogs made anemic by hemorrhage and maintained on a whole cow's milk diet. Potter, Elvehjem and Hart ('38) had already shown that nutritional anemia induced in growing dogs by milk feeding is curable by iron and copper therapy. They had also demonstrated the need for copper with iron for maximal hematopoiesis following hemorrhage. We have attempted to answer experimentally several questions raised by their work.

The specific deficiency of iron and copper obtained in dogs on a milk diet is far less complicated than the partial deficiencies of protein (Robscheit-Robbins and Whipple, '37), individual amino acids (Whipple and Robscheit-Robbins, '37), riboflavin (György, Robscheit-Robbins and Whipple, '38), iron (Hahn and Whipple, '36), and copper (Elden, Sperry, Robscheit-Robbins and Whipple, '28; and Sturgis and Farrar, '35), which exert a deterrent effect to normal hemoglobin building in the salmon-bread diet used by Whipple and his co-workers in their well-known anemia experiments with dogs.

In their work liver supplies all of the individual deficiencies of the salmon-bread diet and produces maximal hemoglobin building, whereas compensation of any individual deficiency, such as riboflavin, for instance, results in only a partial effect. In our work we have felt confident that raw milk supplied all essentials in at least fair amount, except known mineral elements, and that the addition of liver would not enhance its capacity for supporting hemoglobin production appreciably above that obtained by adding the deficient mineral elements. The following experiment was conducted to test this theory.

EXPERIMENTAL

Six mature litter mate collies were made anemic by phlebotomy. The dogs had been on an exclusive milk diet since birth and had overcome one nutritional anemia, or a nutritional anemia followed by a single hemorrhagic anemia, through iron and copper therapy. The amount of blood drawn in order to lower the hemoglobin level to 6–7 gm. per 100 cc. of blood ranged from 400 to 1000 cc. and was proportional roughly to the size of the dog. Bleedings were made about twice weekly for 5 weeks when the dogs were considered to be in a stable state of anemia. This proved to be the case, since during the subsequent 4-week period when no therapy was given, increases in hemoglobin level were uniformly small and the calculated hemoglobin production was negligible (table 1). Blood volume was assumed to be 8% of the body weight in calculating total hemoglobin.

Dogs 1 and 7 were fed 100 gm. daily of whole dried liver which was sufficient to supply 3 mg. of copper and 100 mg. of iron by analysis. They showed the expected response, arriving at normal levels in 4 weeks. The calculated hemoglobin production was optimal by all standards. Dog 5 which was fed adequate iron (30 mg.) and copper (3 mg.) daily showed equally rapid regeneration and the calculated hemoglobin production per kilogram of body weight was approximately equivalent to that obtained in the case of liver therapy; in three instances, namely dogs 3, 4, and 6, where iron alone was

fed the increases in hemoglobin were slight and the calculated hemoglobin production only about one-third that obtained with liver or with iron and copper. Dog 4, after receiving iron alone for 4 weeks, was given copper in addition to iron. The rate of hemoglobin building trebled in the succeeding 4-week period.

The per cent of iron utilized for hemoglobin formation was calculated from the iron ingested and the iron which appeared

TABLE 1

Hemoglobin production in dogs after hemorrhage

DOG	WEIGHT IN KILO-GRAMS	Hb IN GM./100 CC.	CALCULATED TOTAL Hg. IN DOG	DAILY SUPPLEMENT TO MILK DURING 4-WEEK PERIOD	TOTAL Hb PRODUCED IN 4 WEEKS	PER CENT Fe USED IN Hb
	At end of 4-week period					
1	12.3	7.6	75		11	
	14.0	15.4	172	100 gm. whole dry liver*	97	
7	8.9	6.7	48		5	
	10.2	15.0	122	100 gm. whole dry liver	74	
4	14.8	7.1	84	30 mg. Fe	40	
	16.5	15.6	206	30 mg. Fe + 3 mg. Cu	122	16
5	10.9	6.8	59		1	49
	11.6	14.3	133	30 mg. Fe + 3 mg. Cu	74	30
3	12.7	6.9	70		1	
	13.4	9.8	105	30 mg. Fe	35	14
6	9.7	6.8	53		5	
	11.1	8.8	78	30 mg. Fe	25	10

* Vacuum-dried hog liver prepared by Wilson and Company was used. $MnCl_2$ was supplied equal to 1 mg. Mn per dog per day during the experiment.

as new hemoglobin over the 4 weeks of therapy (1 mg. Fe ∽ 0.294 gm. Hb). In the cases where iron alone was fed, utilization to build hemoglobin was not above 16% while in the two instances where copper supplemented iron the per cent utilization was two and three times as great. No correction was made for the small amount of iron supplied by the milk.

Further work has shown that iron utilization is practically nil when copper stores in the animal are reduced to a sufficiently low level. Also the amounts of iron fed in this experiment are considerably above the minimal levels which were

later found to promote rapid hematopoiesis. Both of these factors in this experiment actually operate to minimize the importance of copper.

DISCUSSION

The difference between our technique and that used by Whipple and co-workers should be borne in mind in evaluating these results. In our work the effect of hemorrhage is superimposed on the effect of dietary conditions which make for an iron and copper deficiency anemia only. The diet appears to supply other essentials in good amount. In the Whipple technique a state of anemia is produced prior to therapy and maintained during therapy by continued bleeding, thus imposing continued demands on the diet for blood-building supplies. Both techniques in certain ways simulate the condition of the commonly occurring hypochromic anemias of infancy, blood loss and pregnancy, and chlorosis. The abnormally great demand placed on the diet for all blood building materials by the Whipple technique has been best met by liver therapy. This has been true because the diet used was deficient in several factors and because liver is notoriously rich in all of the many factors which have been in some way related to the hematopoietic function. The part played by each individual factor contained in liver, such as iron, copper, vitamin B_6, and riboflavin must be quite separate and distinct in the complex chain of reactions leading to the in vivo synthesis of hemoglobin. Study of the role of the factors thus far known to play a definite part in hematopoiesis would seem to be best approached by use of diets deficient in only one or two such factors. Milk is admirably suited as a diet deficient in iron and copper, and in cases where iron and copper are supplied it may prove equal to liver in supplying organic factors.

Iron utilization in dogs on a whole milk diet is very complete and under certain conditions may approach 100%. Although Whipple's dogs obtain about 20 mg. of iron per day, calculated utilization of this iron to build hemoglobin is quite low. This suggests that iron utilization is dependent not so

much on the amounts of iron fed, but on the completeness of the diet with regard to other factors influential in the metabolism of iron. These points will be discussed more fully in a subsequent publication.

Presumptive evidence that a deficiency of cobalt in dogs on a whole milk diet may operate at times to retard normal hematopoiesis even in presence of iron and copper has been obtained in this laboratory. In any case the requirement for cobalt must be very small, considerably less than 0.1 mg. per day. In the experiments cited no evidence of a cobalt deficiency was noted.

SUMMARY

These data clearly support the essentiality of copper with iron for hemoglobin production in dogs and present evidence that whole milk with iron and copper can successfully meet the great increase in demand for blood-forming elements occasioned by severe blood loss.

On a whole milk diet regeneration of hemoglobin was no more rapid with liver therapy than with the iron-copper treatment.

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THE EFFECT OF FERRIC CHLORIDE ON THE UTILIZATION OF CALCIUM AND PHOSPHORUS IN THE ANIMAL BODY

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There have been only a limited number of investigations on the problem of the effect of iron salts on the utilization of calcium and phosphorus. Waltner ('27) showed that adding 2% of reduced iron to McCollum's stock diet produced rickets in rats in 4 weeks. The blood serum of the rachitic rats showed low phosphorus but normal calcium, a condition similar to that found in human rickets. Cox, Dodds, Wigman and Murphy ('31), using guinea pigs and rabbits as experimental animals, showed that aluminum or ferric salts added to rations in such amounts that the aluminum or ferric ion was in excess of the phosphorus ion brought about drastic lowering of the bone ash and blood phosphorus. These investigators suggest that the reduction was due to the precipitation of alimentary phosphorus as unabsorbable ferric or aluminum phosphate. Using rats and adding ferric chloride to a non-rachitogenic diet, Brock and Diamond ('34) produced rickets of about the same degree of severity as is produced on the Steenbock rachitogenic diet. By substituting ammonium chloride for ferric chloride and finding that no rickets occurred, they showed that the ferric rather than the chloride ion was the causal factor. Deobald and Elvehjem ('35), working with chicks, demonstrated that the addition of large amounts of ferric citrate or aluminum sulfate to a diet which had been shown to be adequate for normal growth and bone formation brought about severe rickets in 1 or 2 weeks. Bone ash was reduced

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[1] Died January 28, 1940.

almost 50% and the phosphorus in the blood serum was markedly lowered. These investigators suggest a possible danger from the use of large doses of iron in the treatment of hypochromic anemia. Day and Stein ('38) reasoned that if the addition of iron affected phosphorus metabolism, the addition of phosphorus ought to affect iron metabolism. They studied the effect upon hematopoiesis of different levels of calcium, phosphorus, iron and vitamin D in the diet. On diets low in calcium or iron, or high in phosphorus, rats developed anemia and polycythemia. Adding large amounts of iron chloride or calcium carbonate prevented the development of the anemia, but ferric phosphate and calcium phosphate were ineffective. The explanation offered for these results is that phosphorus combines with iron and interferes with its assimilation, but that, if sufficient calcium is present to combine with the phosphorus, the iron is free for assimilation. It is in this sense, Day and Stein think, that calcium is "a sparer of iron."

Two experiments have been reported that are not in line with the conclusions of Day and Stein. Kletzien ('38) reported that various calcium salts interfered with, rather than aided, storage of iron in young rats and that rats receiving phosphoric acid utilized more iron than controls receiving an equivalent amount of phosphorus as tricalcium phosphate. Shelling and Josephs ('34) also reported that calcium hindered iron utilization in rats. Further investigation concerning the metabolism of calcium, phosphorus and iron seems timely. The present experiments offer evidence of the detrimental effect of iron salts when added to an adequate synthetic diet under conditions of controlled food intake.

EXPERIMENTAL

In the first experiment two groups of rats, properly matched as to age, sex and weight, were used. One group was fed a standard, artificial diet, and the other the same diet supplemented with enough ferric chloride to combine with one-half the amount of phosphorus present in the ration. A group was

started in which enough ferric chloride was added to combine with all the phosphorus, but these animals developed a severe anorexia and lost weight so rapidly that it was impossible to use them for experimental purposes. The rats used were of good nutritional stock. They were placed on the diet at 22 days of age and all animals used weighed between 40 and 45 gm. at this time. Each group consisted of six males and six females. The animals were placed in separate galvanized iron cages with raised bottoms to prevent coprophagy.

The diet used consisted of:

	Per cent
Casein, unpurified	20
Cornstarch	56
Butterfat	8
Salt mixture (Osborne and Mendel)	4
Yeast, dried	10
Cod liver oil	2

Food intake for the two groups was kept the same by using the animals on the supplemented diet as controls since they had poorer appetites than those on the unsupplemented ration. Rats were weighed at 4-day intervals and adjustments in the quantity of food given were made at the weighing periods. Calculations at the end of the period showed that difference in food intake amounted to less than 0.5 gm. per day per rat. At the end of 3 weeks four animals, two from each group, were killed and analyzed for total ash, calcium and phosphorus. Results showed that a definitely lower ash content for the experimental rats had already developed and it was decided to terminate the experiment at the end of 1 month. At this time the rest of the animals were killed with ether, the digestive tracts removed and each animal analyzed separately for total ash, calcium and phosphorus. The percentages of total ash, calcium and phosphorus were then calculated on net body weight. Ashing was done in weighed silica dishes; a modification of McCrudden's method was used in determining calcium and the method of The Association of Official Agricultural Chemists for phosphorus.

Table 1 gives the data obtained. This table shows that in spite of careful control of food intake, rats on the unsupplemented diet made greater weight gains than did those on the supplemented ration. This greater growth, in the case of the females, was made in spite of a lower food intake.

Total ash. As will be seen from table 1 the bodies of female rats kept on the synthetic diet supplemented with ferric chloride contained on the average about 0.9 gm. less total ash than did the bodies of female rats on the unsupplemented diet; the difference in the case of male rats amounted to 1.2 gm. This involves reductions of 20.0% and 25.5%, respectively. On the other hand, the percentage of total ash, based on net body weight, varied surprisingly little in the two groups of animals. This lack of variation is partly explained by the greater weight of the animals on the unsupplemented diet, but it should be remembered that this greater weight was attained without increase in mineral intake and would therefore indicate greater utilization of minerals. Variation in the amount of total ash in the individual animals of the same sex and on the same diet was not large and was not correlated directly with body weight.

Calcium. Reference to table 1 shows that as a result of the addition of ferric chloride there was a decrease in the calcium content of 0.29 gm. in bodies of the females and 0.36 gm. in the bodies of males or 23.9 and 28.0%, respectively. The average calcium, based on net body weight, in the bodies of female rats on the unsupplemented diet was 0.96%, and on the supplemented diet was 0.84%; for males, it was 0.91% and 0.81%, respectively. Individual variations in the calcium content of the animals in each group were small.

The decreases obtained in this experiment may be compared with those obtained by Sherman and Booher ('31) in an experiment in which the calcium content of the diet was varied from 0.16 to 0.32%. Sixty-day-old male rats on a diet containing 0.16% calcium had a body content of 0.7%, while those on a diet containing 0.32% calcium had a body content

TABLE 1

The effect of supplementing the diet with ferric chloride for a 30-day period on net body weight of rats, and total body ash, calcium and phosphorus

	RAT NO.	UNSUPPLEMENTED DIET							SUPPLEMENTED DIET						
		Net body weight	Total body ash		Total body calcium		Total body phosphorus		Net body weight	Total body ash		Total body calcium		Total body phosphorus	
		gm.	gm.	%	gm.	%	gm.	%	gm.	gm.	%	gm.	%	gm.	%
Females	1	133	4.412	3.61	1.209	0.91	0.818	0.62	—	—	—	—	—	—	—
	2	122	4.542	3.41	1.232	1.00	0.818	0.67	—	—	—	—	—	—	—
	3	124	4.524	3.64	1.226	0.99	0.804	0.65	—	—	—	—	—	—	—
	4	121	4.385	3.62	1.218	1.00	0.810	0.67	—	—	—	—	—	—	—
	5	130	4.379	3.36	1.186	0.91	0.786	0.61	—	—	—	—	—	—	—
	11	—	—	—	—	—	—	—	110	3.632	3.30	0.946	0.86	0.658	0.60
	12	—	—	—	—	—	—	—	99	3.236	3.26	0.870	0.88	0.584	0.60
	13	—	—	—	—	—	—	—	118	3.634	3.15	0.937	0.81	0.666	0.58
	14	—	—	—	—	—	—	—	109	3.485	3.19	0.911	0.84	0.636	0.58
	15	—	—	—	—	—	—	—	120	3.786	3.15	0.955	0.80	0.684	0.57
	Average	126	4.448	3.53	1.214	0.96	0.807	0.64	111	3.555	3.21	0.924	0.84	0.646	0.59
	Decrease from unsupplemented diet in per cent:									20.0	0.32	23.9	0.12	20.0	0.05
Males	6	141	4.756	3.37	1.278	0.91	0.866	0.61	—	—	—	—	—	—	—
	7	141	4.811	3.41	1.276	0.91	0.877	0.62	—	—	—	—	—	—	—
	8	143	4.803	3.36	—[1]	—	0.869	0.61	—	—	—	—	—	—	—
	9	141	4.676	3.31	1.286	0.91	0.856	0.61	—	—	—	—	—	—	—
	10	145	4.824	3.33	1.293	0.89	0.874	0.60	—	—	—	—	—	—	—
	16	—	—	—	—	—	—	—	110	3.482	3.16	0.897	0.81	0.629	0.57
	17	—	—	—	—	—	—	—	117	3.673	3.08	0.967	0.88	0.677	0.58
	18	—	—	—	—	—	—	—	116	3.636	3.13	0.937	0.81	0.652	0.56
	19	—	—	—	—	—	—	—	107	3.239	3.02	0.845	0.79	0.585	0.55
	20	—	—	—	—	—	—	—	120	3.747	3.12	0.968	0.81	0.696	0.58
	Average	142	4.774	3.36	1.283	0.91	0.868	0.61	114	3.555	3.10	0.923	0.81	0.648	0.57
	Decrease from unsupplemented diet in per cent:									25.5	0.26	28.0	0.10	25.3	0.04

[1] Material lost.

of 0.87%. The difference of 0.17% was considerably larger than the difference of 0.10% obtained in the present experiment by adding ferric chloride to the diet. However, with female rats our difference of 0.12% was somewhat closer to the 0.16% obtained by Sherman and Booher.

Phosphorus. Data given in table 1 show that there was a decrease in phosphorus content of 0.16 gm. and 0.22 gm. in the bodies of female and male rats, respectively, as a result of the addition of ferric chloride to the diet. The corresponding percentage decreases were 20.0 and 25.3. The percentages of phosphorus in the bodies of female rats on the unsupplemented and the supplemented diets were 0.64 and 0.59, respectively. Corresponding figures for males were 0.61 and 0.57. The decreases in the percentage of phosphorus were not as striking as the decreases in the percentage of calcium. Just why calcium metabolism should be more drastically affected than phosphorus metabolism is a matter of conjecture. As was the case with calcium, individual variations in phosphorus content within each group were small.

In regard to total ash, calcium, and phosphorus, it may be. pointed out that differences in the net body weight of animals in the two groups tended to minimize the detrimental effect of ferric chloride on calcium and phosphorus metabolism when expressed in percentage of net body weight; that is, the percentage decreases were quite small. Because of the controlled food intake, it is thought that differences in total ash, calcium, and phosphorus represent a truer picture than percentage differences. On the same calcium and phosphorus intake, much smaller amounts of these minerals were deposited in bodies of animals whose diet was supplemented by ferric chloride than in the bodies of animals whose diet was not so supplemented. It is probable that the interference with calcium and phosphorus metabolism was a causative factor in the marked anorexia and consequent poor growth of the animals on the ferric chloride supplement.

Effect of reducing cod liver oil. Because of the influence of cod liver oil on calcium and phosphorus metabolism, we

thought that it would be interesting to repeat the experiment, using smaller amounts of cod liver oil in the diet. It was also decided to adopt the pair-mate method in order to control the food intake even more accurately. In this method two animals of the same age, sex and weight are selected at the beginning of the experiment as pair-mates. The food intake for each day is carefully computed, and the animal on the smaller intake acts as control in determining the amount of food allowed his pair-mate. In this case it was, of course, the animals on the supplemented diet which acted as controls for those on the unsupplemented diet.

Four pair-mates, two on the supplemented and two on the unsupplemented diet, were studied over a period of 30 days;

TABLE 2

Net body weight of pair-mate rats

RAT	UNSUPPLEMENTED DIET NET BODY WEIGHT	RAT	SUPPLEMENTED DIET NET BODY WEIGHT
	gm.		*gm.*
2	135	8	104
3	130	9	94
4	126	10	98
5	130	11	101

the diet, in this case, contained only one-half the amount of cod liver oil as in the previous experiment. Net body weights are given in table 2. Again the rats on the unsupplemented diet made better weight gains than those on the supplemented.

Two animals on the unsupplemented diet were ashed together in the same silica dish, care being taken to select those of similar gains in weight and having similar food-intakes. The same procedure was followed with their pair-mates. Results are given in table 3.

In the previous experiment the average amounts of total ash, calcium, and phosphorus in the bodies of male rats on the unsupplemented diet were 4.774 gm., 1.283 gm., and

0.868 gm., respectively. When the cod liver oil was reduced by one-half, comparable amounts were 3.996 gm., 1.057 gm., and 0.745 gm. (average of nos. 2, 3, 4 and 5 in table 3). Thus, it will be seen that reducing the cod liver oil decreased the amounts of total ash, calcium and phosphorus in the body. This would be expected, since cod liver oil is known to improve retention of calcium and phosphorus. The percentage differences, however, between the animals fed the supplemented and unsupplemented diet were smaller when the cod liver oil was lowered. In the previous experiment

TABLE 3

Effect of ferric chloride on total ash, calcium, and phosphorus in bodies of pair-mate male rats

RATS		TOTAL ASH	CALCIUM	PHOSPHORUS
		gm.	*gm.*	*gm.*
Controls	2 and 3	8.010	2.119	1.498
Pair-mates	8 and 9	6.499	1.683	1.183
Decrease		1.511	0.437	0.315
Per cent decrease		18.9	20.6	21.0
Controls	4 and 5	7.972	2.109	1.483
Pair-mates	10 and 11	6.362	1.704	1.198
Decrease		1.610	0.405	0.285
Per cent decrease		20.2	19.2	19.2

the average percentage differences in total ash, calcium and phosphorus in the bodies of male rats were 25.5, 28.0, and 25.3, respectively. When the cod liver oil was reduced by one-half, comparable percentages were 19.5, 19.9, and 20.6 (average of decreases in table 3). This would indicate that the effect of adding ferric chloride is less drastic when small amounts of cod liver oil are supplied in the diet. From the standpoint of iron utilization these results indicate that iron might be better utilized when small rather than large amounts of cod liver oil are added to the diet. Less of the phosphorus is evidently bound as ferric phosphate; therefore more iron should be available.

SUMMARY AND CONCLUSION

A comparison of the amounts of total ash, calcium, and phosphorus in the bodies of animals on an unsupplemented diet with the amounts in bodies of animals on a diet supplemented with enough ferric chloride to combine with one-half the phosphorus of the diet was made, and it was shown that the addition of ferric chloride resulted in a considerable reduction in the amounts of total ash, calcium, and phosphorus at the end of 30 days. Similar experiments with like results have been reported in the literature, but in no other study has the food intake of the two groups of animals been equalized so that mineral intake of both groups was the same. In the present investigation the food intake of all animals has been kept approximately the same. In spite of this equalized food intake, animals on the unsupplemented diet gained more weight, and had larger amounts of calcium, phosphorus, and total ash deposited in their bodies at the end of the experimental period than animals with the ferric chloride supplement.

Although the analysis of each animal was carried out separately, discussion of results was based on group averages. Sex differences were not considered to be of such magnitude as to warrant separate discussion. Comparisons were made of percentage and total calcium, phosphorus, and ash in the bodies of both groups of animals. It has been pointed out that differences in net body weight of animals in the two groups tend to minimize the detrimental effect of ferric chloride on calcium and phosphorus metabolism, when expressed in percentage of body weight. Because of controlled food intake, it was thought that differences in total ash, calcium, and phosphorus represent a truer picture than percentage differences in arriving at a conclusion as to the effect of iron on calcium and phosphorus metabolism. Results of this experiment indicate that ferric chloride has a detrimental effect on calcium and phosphorus metabolism.

When the amount of cod liver oil in the diet was reduced by 50% the addition of ferric chloride resulted in a less drastic lowering of body calcium and phosphorus.

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INVESTIGATION OF THE VITAMIN C CONTENT OF FLORIDA FRUITS AND VEGETABLES

I. EFFECTS OF MATURATION AND OF COLD STORAGE ON THE VITAMIN C POTENCY OF ORANGES AND GRAPEFRUIT [1]

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The development of quick chemical methods for the determination of vitamin C has stimulated the publication of considerable work dealing with its natural distribution. Citrus fruits, long known as an important source of vitamin C in the human dietary, have received their share of attention. Many investigators (Harris and Ray, '33; Bacharach, Cook and Smith, '34; Guerrant, Rasmussen and Dutcher, '35; Daniel, Kennedy and Munsell, '36) have published results indicating considerable variability in the vitamin C content of citrus fruits.

This paper is a report on the effect of maturation, location of grove, and cold storage on the vitamin C concentration of oranges. Some studies of the effect of storage on the vitamin C potency of grapefruit are also included. Beacham and Bonney ('37) published estimations of the vitamin C content of Florida citrus fruits as affected by such factors as variety and root stock differences, picking dates and geographic location, but did not attempt to draw any conclusions from their variable and extensive data. Their samples, like ours, were

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[1] Published with approval of the director of the Florida Agricultural Experiment Station.

gathered during the season of 1936 and 1937 and in one case a comparison of their results with ours from the same grove is possible.

METHODS

Four dozen or more fruit were picked and within a day taken to the laboratory for analysis. The fruit was then graded according to size and color. Obviously off-size, whether small or large, or off-color fruit was either discarded or made up in separate samples. A few determinations on off-color fruit are reported; the number of fruit in these samples ranged from three up to the minimum requirement of twenty-four for a satisfactory sample.

The fruit was weighed, peeled (using a glass knife), and the juice expressed through cheese-cloth in a porcelain-block press, care being taken that the sample at no time came in contact with metals. The juice volume represented the easily expressible juice. Weights of the fruit and skin and of the volume of juice were recorded, and acid and vitamin C determinations were made on the juice.

Total acidity was determined using phenolphthalein as an indicator. The vitamin C was determined by titrating a milliliter of juice, to which a milliliter of 10% trichloracetic acid had been added (Birch, Harris and Ray, '33) with Tillmans' indicator (0.2% aqueous solution of sodium 2,6,dichlorobenzenone-indophenol, Eastman) (Tillmans, '30). The indicator solution was standardized against ascorbic acid.[2] One milliliter of the purified indicator was equivalent to approximately 1 mg. of ascorbic acid.

EXPERIMENTAL

Maturity. In the grove where the fruit was obtained, the major bloom occurred in January and February, and fruits set from these blossoms attained considerable size in October. Sampling, therefore, began the middle of October, and continued thereafter at 14-day intervals until June, 1937. The

[2] Cebione from the Merck Company.

fruits collected came from individual trees and were of three varieties, Parson Brown (early ripening), Pineapple (mid-season) and Valencia (late ripening). The Parson Brown oranges in this grove were picked for market in November, and the Pineapple and Valencia during January and March, respectively.

The data on the effect of maturation on both acid and vitamin C content of Parson Brown, Pineapple and Valencia oranges are presented in spot diagrams 1, 2 and 3. For purposes of comparison, the data of Beacham and Bonney ('37) are also given.

There was a decrease of acidity in all the oranges as maturation advanced, the rate being about the same for the Parson Brown and Pineapple, and a little greater for the later-ripening Valencia. The decrease continued without apparent change in rate as long as the oranges remained on the tree.

The distribution of points on the graphs shows that the vitamin C concentration during the 6-month period of observation remained surprisingly constant. Such tendencies as might be suggested are masked by individual variation, and more data would be needed to establish them.

Unripe green oranges taken from different parts of the tree—top, bottom, inside and outside branches—showed little variation in either acid or vitamin C content, but after general crop yellowing both the Parson Brown and Pineapple oranges that did not color well produced juice that was judged of lower quality and was usually lower in vitamin C. However, the poorly-colored Valencias might be either higher or lower in content of acid and vitamin C, the difference in this case probably being due to the difficulty in separating the "off" colored fruits from those showing a normal "greening up." The variability in vitamin C concentration among oranges obtained from different sources may be considerable (table 1); among like-appearing oranges from the same tree it is very much less (figs. 1, 2 and 3). The parallelism between our data and those of Beacham and Bonney ('37; crosses in figs. 1 and 2) is noteworthy and suggests that analyses of oranges

from the same tree show a considerable degree of reproducibility; also, that analyses of oranges of the same variety from neighboring trees may or may not show equal vitamin C concentration, but that the value obtained is probably characteristic for the tree.   The oranges in these and in Beacham and

TABLE 1

Concentration of vitamin C in citrus fruit grown in several localities, and the effect of storage

| VARIETY | LOCATION IN FLORIDA | VITAMIN C IN MILLIGRAMS PER 100 ML. | |
		As obtained	After storage 1 month at 42°F.
Pineapple orange	North	55	68
	Central	65 [1]	52
	Central	75	91
	Central	53	..
	Central	60	..
	East coast	65 [1]	53
	East coast	68	79
	East coast	70	81
Florida seedling grapefruit	Central	47	54
	Central	38	..
	Central	46	..
	Central	56	..
	East coast	44 [1]	41
	East coast	40	45
	East coast	38	48
Valencia orange	North	48	
	Central	38	
	Central	38	
	Central	36	
	Central	33	
	Central	49	
	Central	48	
	Central	50	

[1] Fruit had been processed before packing.

Bonney's experiments were obtained from the same grove but from different trees.   When considered as a whole no association could be detected between the concentration of vitamin C in the oranges used in these studies and the weight, amount of peel, or volume of juice.

That there is considerable drop in vitamin C concentration at some early stage of development is shown by analyses on samples of green, young Valencias. These weighed less than 10 gm. apiece and the expressed juice gave high titration readings averaging 347 and 335 mg. of vitamin C per 100 gm. for the outer rind and the inner portion of the fruit, respectively. Such values indicate that in these minute fruit there is almost as great a total quantity of vitamin C as there is in a small mature fruit.

Location. Oranges and grapefruit were obtained from the north, central and east coast citrus regions of the state.
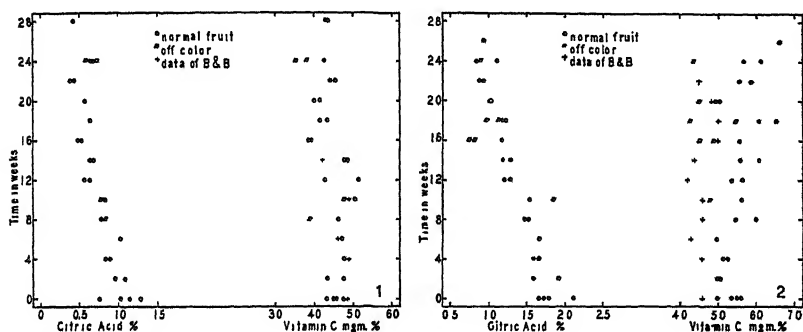
Fig. 1   Changes in citric acid and vitamin C concentration during maturation of the Parson Brown orange between October 16th and April 30th. "Data of B & B" are those of Beacham and Bonney ('37).

Fig. 2   Changes in citric acid and vitamin C concentration during maturation of the Pineapple orange between October 16th and April 2nd. For "data of B & B" see Beacham and Bonney ('37).

These were analyzed according to the procedure outlined, and the average results are given in table 1. The range of values for vitamin C seems to bear no relation to the section in which the fruit was grown. This suggests that climatic or geographical influences within the regions studied were not factors of major importance. This conclusion is supported by the data presented by Beacham and Bonney ('37).

Storage. Pineapple oranges from six different sources and Florida seedling grapefruit from four different sources were put in storage at 42°F. Samples of these fruits were removed

at frequent intervals during a period of 5 months and determinations made upon them according to the outlined procedure.

The data were gathered together in the form of spot diagrams and observations made on the interrelationships between weight, volume of juice, length of storage period, and concentrations of acid and vitamin C. Except for correlations between acid and vitamin C, and between length of storage period and both acid and vitamin C, no associations between the several factors could be noted.
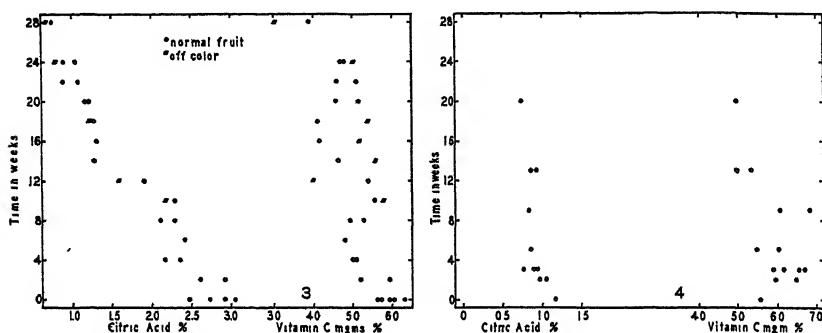
Fig. 3   Changes in citric acid and vitamin C concentration during maturation of the Valencia orange between October 16th and April 30th.

Fig. 4   Changes in citric acid and vitamin C concentration in the Pineapple orange during storage from February 2nd to June 28th.

An example of the data obtained from one crate of Pineapple oranges is given in figure 4. These were oranges picked from the Pineapple tree 16 weeks after the start of the studies of the influence of maturity. A comparison of the data of figure 2 from 16 weeks on with those of figure 4 shows that both the acid and vitamin C concentrations go through about the same changes whether the orange remains on the tree or is put in cold storage.

A small increase in vitamin C concentration after 2 and 3 weeks in storage can be noted in figure 4. This change may not in itself be significant, but seven out of the ten stored · samples of oranges and grapefruit showed variable increases

in vitamin C concentration after storage for 1 month. The extent of these changes, increases up to 26% for seven samples and decreases amounting to 20% for three, is shown in table 1. Examination of the fruit that failed to increase in vitamin C content showed that (contrary to our request) it had been processed before being shipped. Processing may have interfered with normal metabolic changes.

The effect of loss of weight due to drying out of the oranges would not significantly affect these results since Stahl and Fifield ('36) have shown that oranges treated similarly will lose less than 3% of their weight in a month's time.
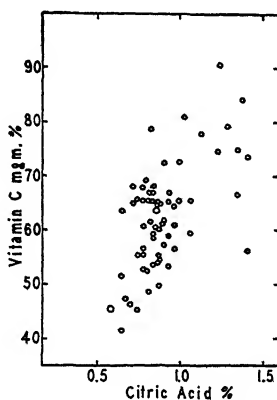
Fig. 5   The relation of citric acid content to that of vitamin C in ripe Pineapple oranges during storage at 42°F. for 5 months.

Figure 5 is a spot diagram depicting the relationship of acid and vitamin C in the Pineapple oranges for the whole storage period of 5 months. Considerably higher values for vitamin C are associated, apparently, with slightly higher acidity values. Among the individual samples that make up these data we find four cases in which there is such an association and two cases in which it is lacking. With the grapefruit, association was obtained in only one case while in three others it was lacking.

There has been a tendency to postulate an association of acid and vitamin C in citrus fruit, partly because of the

fact that, in vitro, acid tends to stabilize vitamin C solutions, and also because of other observations such as those of Lorenz ('36). The data presented in this paper do not deny the possibility of an association but they suggest that any simple association should not be very good, because during both maturation and storage of the fruit fluctuations in vitamin C concentration occur that are unaccompanied by similar changes in acid concentration.

DISCUSSION

The experimental error of a vitamin C determination is easily kept within 2%. The end point of the titration is sharp with citrus juices if the indicator is freshly prepared. Grinding the fruit pulp in a tinned meat-chopper or extracting in a steel press may cause a diminution in titration value, presumably due to contamination with iron, and for these reasons metal contacts in preparing the fruit were avoided.

How accurately the titration procedure measures the vitamin C in citrus juice is still a question. The value obtained has an accuracy at least as great as that determined by the biological procedure, since it has been shown that values secured volumetrically check reasonably well with those obtained by feeding experiments (Tillmans and Hirsch, '33; Harris and Ray, '33).

The cause of the increase noted in vitamin C content of stored fruit was investigated, but no positive conclusions were reached. Hydrolysis of the juice of fresh fruit in 10% phosphoric acid under carbon dioxide produced no change in titration value. Evidently, no ester combination of ascorbic acid is present as was suggested by Reedman and McHenry ('37) in dealing with a similar phenomenon in vegetables; nor could the presence of reversibly oxidized ascorbic acid be demonstrated by following the procedure of McHenry and Graham ('35).

Confusion over the demonstration of reversibly oxidized ascorbic acid may possibly arise in the following way. If the sample has been contaminated with iron or copper, a lowered

titration value may be obtained as noted by Barron, Barron and Klemperer ('36) and others. If the contamination occurs after the addition of acid, the value obtained will be higher if phosphoric rather than any other acid is used. Phosphoric acid tends to protect the vitamin under such conditions by precipitating the heavy metal. In either case if the contaminated samples are reduced with H_2S and titrated, a definite increase in value will be obtained. The increase will be proportional to the quantity of iron (or copper) contamination since ferrous (or cuprous) sulphide is oxidized by the indicator.

SUMMARY AND CONCLUSIONS

Differences in vitamin C content both among samples of one variety and among different varieties of citrus fruit may be very large. Individual trees tend to produce fruit the vitamin C content of which varies only within narrow limits, but these small variations are sufficient to mask any changes occurring during maturation of the fruit. Changes in vitamin C content of oranges during maturation are not large.

Variations in vitamin C content among oranges did not correlate with the different geographical locations in which they were grown.

The vitamin C concentration of oranges and grapefruit may increase during the first few weeks of cold storage; after this, the concentration of vitamin C drops off slowly.

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GREYING OF FUR AND OTHER DISTURBANCES IN SEVERAL SPECIES DUE TO A VITAMIN DEFICIENCY [1]

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THREE FIGURES

Loss of pigmentation in the fur of rats fed diets deficient in one or more of the B vitamins has been observed by various investigators (Bakke, Aschehoug and Zbinden, '30; Gorter, '34, '35; Hartwell, '23; György, '35). Similar changes were seen in rats made anemic by the use of whole milk diets by Keil and Nelson ('31). The latter workers and Gorter ('35) stated that administration of small amounts of copper salts produced darkening of the bleached hair.

Morgan, Cook and Davison ('38) were the first to note that the active substance which prevents and cures the greying is present in the filtrate fraction of the vitamin B_2 complex, that is, remains in the water extract of yeast, liver or rice bran after repeated treatments with fuller's earth have removed vitamins B_1, B_6 and riboflavin. Lunde and Kringstad ('39) have fully confirmed this observation and further have concluded that the "rat growth" and "chick anti-dermatitis" filtrate factors of Lepkovsky, Jukes and Krause ('36) and Elvehjem and Koehn ('35) are not identical with the anti-greying factor.

Whether the greying observed in anemic rats which is cured by copper salts is different in character from that seen in non-anemic rats lacking the anti-grey factor of the B_2 complex remains to be determined. It is possible that both copper and the anti-grey filtrate factor are necessary for maintenance of the color of fur and that deficiency in either will produce depigmentation. The whole milk diet which is used for nutritional anemia production is certainly low in the "filtrate factor" as well as in iron and copper (Jukes and Richardson, '38).

In none of the earlier observations of depigmentation has there been any correlation of the phenomenon with other changes, particularly those of senescence, which might be expected to accompany the greying of the hair. It is clear that the greying must be caused by failure of pigment formation or by destruction of pigment and that the mechanism controlling this function may be damaged. Since both the adrenal and thyroid glands have been thought to be concerned with pigment and hair growth either directly or indirectly through the sex glands, these were examined in the greyed animals.

EXPERIMENTAL METHODS

The basal diet which we have used to produce the greying consists of washed casein [3] 22, salt mixture (Hubbell, Mendel and Wakeman, '37) 2.5, Crisco 9, and sucrose 56.5 parts. The daily supplements per rat were thiamin chloride 10 micrograms, riboflavin 30 micrograms, wheat germ autolysate or eluate equivalent to 0.5 gm. wheat germ, cod liver oil 4 drops. Young rats were placed at weaning on the basal diet supplemented by the thiamin chloride and cod liver oil for 3 to 4 weeks, at the end of which time their growth had ceased. The riboflavin and wheat germ eluate (as source of vitamin B_6) were then given with prompt but usually temporary

[3] We are grateful to the Western Condensing Company of San Francisco which through the courtesy of Drs. P. D. V. Manning and E. L. R. Stokstad provided the supply of washed casein used in this investigation.

resumption of growth. After 56 to 70 days at least half of all such rats began to show the characteristic changes in the fur.

RESULTS

The greying of the fur

The greying was preceded by a change in texture of the fur which became dry and somewhat brittle, a dryness which was also noted in the fur of white rats fed the same deficient diet.
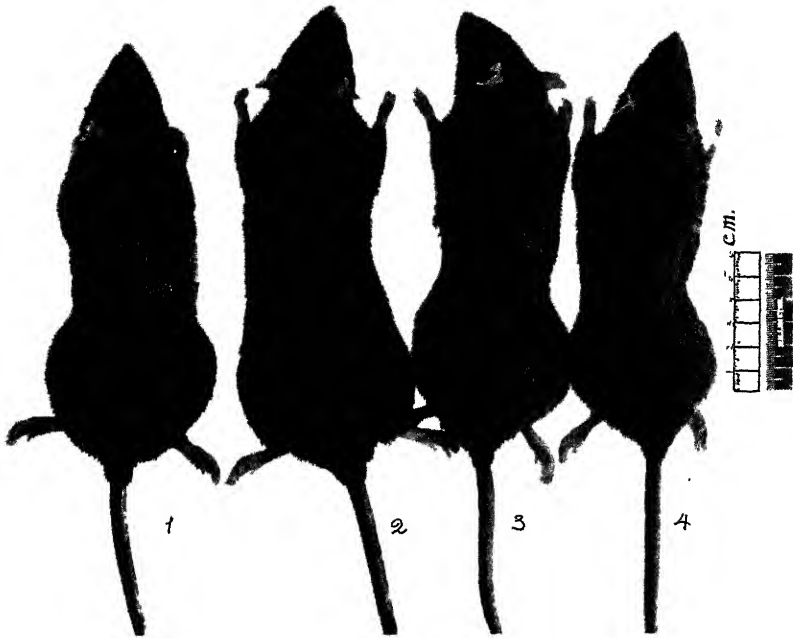
Fig. 1 Typical greying animals of the same litter. Numbers 1, 3 and 4 were fed the anti-grey vitamin-deficient diet; number 2 received the same diet plus the missing substance.

The fur then became a dull mousey brown, metallic and finally grey in color. The mousiness and greyness occurred usually in a definite bilateral symmetrical pattern. The most common pattern was that in which the top of the head and the sides of the body were greyed. Examples of greyed rats are shown in figure 1 along with a normal rat of the same litter.

Some seasonal influence has been seen in the phenomenon, as illustrated in table 1 by the rate of greying of eight groups of rats started on the diet over a period of 2 years. More rapid greying occurred in the later summer and autumn than in winter and spring but there was much variation. Perhaps the seasonal changes in rate of growth of hair may account for this since the depigmentation was found to originate in the follicle and to be observable only as the normal hair was shed. Apparently at least 30 days are required for the new hair growth to affect the color of the hair tips.

TABLE 1

Time required for greying of rat fur due to vitamin deficiency

NUMBER OF ANIMALS	DATE STARTED AFTER PRELIMINARY DEPLETION	DATE WHEN MORE THAN 50% WERE GREY	TIME REQUIRED FOR GREYING
			days
33	May 20, 1937	July 15, 1937	55
12	July 29, 1937	October 7, 1937	70
32	September 23, 1937	December 2, 1937	70
33	December 2, 1937	March 1, 1938	89
37	May 26, 1938	July 21, 1938	56
15	December 21, 1938	March 1, 1939	70
	WITHOUT DEPLETION		
18	October 12, 1938	December 21, 1938	70
35 [1]	February 15, 1939	June 13, 1939	120

Total 215.

[1] Wheat germ diet used.

This was the shortest period in which complete return to normal color was produced by any curative agent. The blue black coloration of the skin of the dark areas in pied rats was also bleached in the process of greying and darkened when cures were effected.

Dermatitis

In addition to greying we have noticed in the cases of chronic insufficiency in older animals a dermatitis which is unlike that due to specific vitamin B_6 deficiency. It is manifested by groups of small lesions located behind the shoulders

and extending partially down the sides (fig. 2). The fur around these lesions is often thin, dry and discolored. No loss of weight accompanies this condition.

Among all the animals regardless of age there often developed also a different type of dermatitis similar in some respects to that in vitamin B_6 deficiency. It was characterized first by a reddening of the nose and a sloughing off of

Fig. 2 Normal and grey rats and those showing the shoulder type dermatitis of the anti-grey vitamin deficiency. Numbers 1 and 4 have shaved patches on the shoulders to show the peculiar type of eruption. This and the ulcers developed mostly in deficient rats which resisted greying.

the fur leaving the skin raw and exuding a moist brownish fluid. This sloughing gradually extends over the face, around the eyes, to the top of the head, to the skin of the abdomen and the legs above the knee and elbow joints. It is important to note that the paws are unaffected usually and that the skin is

wrinkled. Within a short time the animal loses considerable weight and death soon ensues. If, however, filtrate factor is administered, it is possible for the animal to be almost completely cured, within 4 to 12 weeks this including the growth of a new thick pelage, loss of dermatitis and return to previous weight.

Ulcers

In a few of these elderly animals there occurred a severe sloughing of the skin usually beginning first above the base of the tail. The denuded area was moist, inflamed and hemorrhagic, and reminiscent of the lazy leg ulcers observed in humans in the tropics. The slowly spreading ulcer, 2 inches in diameter on one rat, was not cured by administration of as much as 40 micrograms per day of crystalline vitamin B_6.[4] This rat had been fed the filtrate factor deficient diet for 18 months without greying of the fur or interference with normal growth. In November, 1938, the first sign of ulceration began and by March, 1939, the condition had become very severe. At this time over a period of 5 weeks a total of 1.05 mg. crystalline B_6 was injected in gradually increasing doses but without effect upon the ulcer. In May, 1939, a filtrate factor concentrate made from brewer's yeast was fed in amount equivalent to 3 to 5 gm. of yeast daily. Almost at once the ulcer began to dry, scar tissue was formed about the periphery, and by July, 1939, the damage was completely repaired. The vitamin administration was stopped in September, 1939, and 2 months later the ulcer area was once more inflamed.

Another rat, fed the deficient diet from December 6, 1937, when it was 7 weeks old, developed the same type of ulcer in December, 1938. From March 13, 1939, wheat germ oil,[5]

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[4] We are indebted to Dr. S. Lepkovsky for a supply of this material.

[5] The wheat germ oil was tried because another rat with similar ulcer for 7 months had improved when fed whole wheat germ ad libitum for 2 months. Although wheat germ contains some filtrate factor it is not rich in this substance and it was necessary to determine whether the oil phase rather than the B vitamin factors of the germ had the curative effect.

5 cc. per day was fed until April 17, 1939, but without effect. The ulcer continued to spread. This rat was then treated with injections of adrenal cortex extract,[6] 180 D.U. per week, for 12 weeks but without improvement. In December, 1939, the ulcer was still raw and bleeding and the fur had developed snow-white patches, although the normal glossy black color had persisted through nearly 2 years of deficiency.

Relation of growth to greying

The growth of rats on this diet was variable but usually subnormal. In general the rats which greyed early grew less than those which resisted the greying effect. At first we were led by this coincidence to believe that the anti-grey and the ''rat-growth filtrate factor'' were identical but later experience has shown that this is possibly not true. Preparations which produce rapid cure of the greying may allow little increase in growth, and diets upon which excellent growth is obtained may allow early and complete greying. In our earlier experiments in which a particular brand (A) of brewer's yeast, 0.5 gm. per day, was used for positive controls greying was never seen in these animals. Later when another brand (B) of brewer's yeast was substituted, greying of well-growing rats often occurred. This was seen first in animals used for certain protein tests and also in vitamin A assays. Obviously yeast ''B'' was less well endowed with the anti-grey vitamin than yeast ''A'', although equally good growth was obtained with both.

Cures of the greying were effected promptly by preparations which promoted little growth as may be seen in figure 3. The cane molasses and alfalfa extracts induced rapid growth but only late and incomplete cure of greyness, while rice bran and liver filtrates had less effect on growth but caused prompt darkening of the fur. This was strikingly true of the rice bran preparation. The yeast filtrate produced some growth and fairly rapid darkening of the fur.

[6] The adrenal cortex extracts were kindly provided by Dr. Oliver Kamm of Parke, Davis and Company.

The "chick anti-dermatitis factor" which has been said by Jukes ('39) and by Woolley, Waisman and Elvehjem ('39) to be identical in properties with the pantothenic acid of R. J. Williams may or may not be the same as the rat growth factor or the anti-grey factor. This was tested by the following experiment.

A yeast eluate was made by extraction of yeast B (see above) with 50% ethyl alcohol, concentrated, acidified with HCl and treated with fuller's earth repeatedly. The adsorbate, after thorough washing with very dilute HCl, was eluted with dilute ammonium hydroxide, washed several times and the eluate and washings concentrated until 1 cc. was the
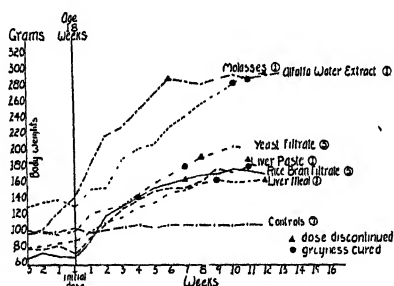
Fig. 3   Relation of anti-greying to growth responses produced by various filtrate preparations.

equivalent of 45 gm. yeast. The concentrate was made strongly alkaline with sodium hydroxide and boiled under reflux for 6½ hours, then cooled and neutralized with HCl. The resulting product, yeast fraction 325-A, contained the equivalent of 26 gm. of yeast per cubic centimeter but little pantothenic acid if this substance is destroyed by such prolonged heating in alkaline medium. This product, supplied by Dr. T. H. Jukes, in 0.1 cc. daily doses rapidly cured the greying and at the same time promoted the growth of young rats which had been in an advanced stage of the anti-grey deficiency condition. The product had however retained a mild anti-dermatitis efficacy when tested on chicks.

Pure beta-alanine, which was shown by Woolley, Waisman and Elvehjem ('39) to be part of the pantothenic acid molecule, when fed in doses of 100 to 250 micrograms daily to grey rats increased the severity of the symptoms and caused early death. This is similar to the effect of nicotinic acid.

Specificity of the anti-grey vitamin

Various other substances have been tried both as preventives and cures particularly for the greying but also for the dermatitis, and to determine the relation of the other vitamins of the B complex to these conditions. The following facts were brought to light.

Nicotinic acid or amide does not cure or prevent greyness, and in excess apparently exaggerates the dermatitis of the face and abdomen and hastens the death of the animals.

Vitamin B_1 or riboflavin in doses of 200 to 250 micrograms daily, vitamin B_6, vitamin B_6 and riboflavin together in excess, wheat germ, wheat germ oil, and copper (as $CuSO_4$) have all been found to be ineffective in curing or preventing the greying and the dermatitis.

Yeast, yeast filtrate, concentrated acid yeast eluate, liver meal, liver paste, liver filtrate, rice bran filtrate, crude cane molasses and alfalfa extract cure the greyness at varying rates of speed. Yeast and rice bran filtrates cure all types of dermatitis produced by the deficiency.

An illustrative experiment to determine the effect upon the greying by the various constituents of the vitamin B_2 complex was made upon forty-four weanling black rats from six litters. All of these animals were depleted by 4 weeks of subsistence on the usual basal diet with vitamin B_1 and cod liver oil supplements.

After 4 weeks, growth had practically ceased. The rats were then divided into six groups with daily additions to their diets for 6 weeks as follows: (1) no addition; (2) 0.5 gm. brewery yeast; (3) riboflavin, 30 γ; (4) wheat germ (acetone extracted) autolysate equivalent to ½ gm. wheat germ as source of vitamin B_6; (5) yeast filtrate free from riboflavin and vita-

min B_6, equivalent to 1 gm. yeast; (6) riboflavin, B_6 preparation and yeast filtrate in the amounts mentioned for the preceding groups. The plan was to produce each type of double deficiency for 6 weeks, then supply one of the missing vitamins to some of the animals of each group, thus providing also single deficiencies for 6 weeks. Finally for a third 6 weeks' period there was given to some rats of each group the third missing factor. At the end of 18 weeks after depletion there were therefore a few rats showing all the possible double and single B_2 deficiencies as well as some which had been cured successively of these deficiencies.

The groups given yeast and all three of the B_2 factors, i.e., groups two and six, appeared normal at all times although group six did not grow satisfactorily. The other four groups which were deficient in either two or three of the B_2 vitamins made little or no growth but no greying of fur occurred except in the rats receiving the B_6 preparation. In the second 6 weeks' period when there were single as well as double deficiency groups greying occurred only in the animals receiving riboflavin and B_6. In the third 6 weeks' period in which some of each deficient group received one or both of the missing factors, greying again occurred only in those groups which received B_6 only, or B_6 and riboflavin. Cures were seen in each of the greying groups to which filtrate preparations were administered during either the second or third periods. At the end of the eighteenth week, of the sixteen surviving rats which had not received filtrate factor, ten were completely grey. Five of the others were suffering from B_6 deficiency and only one which received B_6 but no filtrate factor had normal pigmentation.

Double deficiencies were manifest in several of these animals. The mole-like fur and denuding appeared along with the greying in several of those which were deficient in both riboflavin and filtrate factor. The rats which were deprived of both B_6 and riboflavin, in several instances survived long enough to exhibit simultaneously fur and skin symptoms typical of both deficiencies. It was not possible usually to obtain

in the same animals the acrodynia of B_6 deficiency and the greying effect of filtrate factor deficiency. However, several animals have been observed which have developed simultaneously the triple deficiency, i.e., acrodynia of vitamin B_6 deficiency, ariboflavinosis and greying of the fur.

The damage to the adrenal glands

Histological studies of rats fed the deficient diet were made, with special attention given to the condition of the adrenals, thyroid, skin and gonads. Parallel examination was also made of litter mates on the same diet but deficient in riboflavin, or vitamin B_6 instead of filtrate factors. The chief distinctive change found was in the adrenal glands of the filtrate factor deficient animals. These microscopic studies will be reported in full elsewhere.

In animals which had greyed, the zona reticularis was degenerated, with heavy deposits of yellow pigment and connective tissue and excess vascularity.[7] These conditions were not marked in the deficient animals which had not greyed and were largely remedied in those which had been cured by administration of the anti-grey preparations.

The thyroids of the grey animals were also damaged, with no serration of colloid which stained quite uniformly instead of irregularly as in the normal thyroid. Some acini were totally filled with epithelial cells, thus indicating relative inactivity of the gland. The more advanced the greying of the fur, the greater was the abnormality of the thyroid.[8]

No such changes were seen in the adrenals and thyroids of rats fed diets deficient in riboflavin or vitamin B_6.

Loss of the elastic layer of the skin and failure of spermatogenesis were also noted in the greyed rats.

[7] A preliminary note on this phenomenon appeared in Science, vol. 89, pages 565–566, June 16, 1939.

[8] Dr. Jesse L. Carr of the Pathology Department, University of California, School of Medicine, gave us indispensable assistance in the preparation and interpretation of the tissue studies.

Treatment with adrenal cortex extract, adrenalin and thyroid extract separately or in various combinations was tried as means of producing a cure of the greying with the results shown in table 2. The adrenal cortical extract was most effective in curing the condition but thyroid was also effective. The adrenalin appeared on the whole to be of indifferent value, although a sudden and complete greying occurred in several animals 2 or 3 weeks after the adrenalin treatment was discontinued. In all cases the darkening of the hair was accomplished far more slowly with the cortin and thyroid extract than with potent filtrate preparations.

Effect on lactation [9]

In the absence of the filtrate factor or factors failure of lactation occurs invariably in rats. Adult female rats which had borne and successfully reared a first litter were placed on the experimental diet on the day of mating or on the day of littering. Good records were secured with the yeast supplement, with all three vitamin additions, that is, B_6, riboflavin and filtrate factor, poorer growth with B_6 deficient and riboflavin deficient diets but no survival of young on filtrate factor deficiency except in a few cases when the deficient diet was given only after the young were born.

Two specific lactation-controlling vitamins, one found in liver and one in yeast, have been postulated by Nakahara, Inukai and Ugami ('38). Certainly in our experiments success in lactation was possible with a diet containing the filtrate factor preparation and failure resulted on the same diet and identical supplements but without the filtrate factor or factors.

Experiments with guinea pigs

An attempt was made to produce the symptoms of the deficiency in guinea pigs. This was difficult because of the poor response of this species to synthetic diets. However,

[9] The work on lactation was done by Miss Lura M. Morse. A full report of this will appear later.

TABLE 2

Summary of curative treatment of greyed rats with substances other than members of the vitamin B complex

RAT NO.	AGE AT BEGINNING OF TREATMENT	MATERIAL USED IN THE TEST	FIRST LEVEL OF DOSAGE		SECOND LEVEL OF DOSAGE		REMARKS
			Per week	For	Per week	For	
	weeks			*weeks*		*weeks*	
192	14	Cortin	40 DU[1]	13	180 DU	10	Grey decreased. Died after 23 weeks of treatment
250	14	Cortin	40 DU	6	80–180 DU	15	Grey disappeared after 6 weeks
6110	16	Cortin	180 DU	6			Grey disappeared
254	14	Cortin Thyroid	40 DU 1–2 grains	12	180 DU 2 grains	10	Grey disappeared after 15 weeks
197	14	Cortin Thyroid	40 DU 1–2 grains	12	180 DU 2 grains	10	Grey decreased. Died after 22 weeks of treatment
204	14	Cortin Adrenalin	40 DU 1.5 cc.	1			Died
252	14	Cortin Adrenalin	40 DU 1.5 cc.	12	180 DU 1.5 cc.	9	Grey disappeared after 8 weeks, then brown color appeared
257	14	Adrenalin	1.2 cc.	8	1.5 cc.	12	Some darkening after 20 weeks, then greyed suddenly
190	14	Adrenalin	1.2 cc.	8	1.5 cc.	12	Darkened after 17 weeks, then greyed suddenly
202	14	Thyroid extract	1 grain	5	2 grains	17	Grey disappeared after 15 weeks
189	14	Thyroid extract	1 grain	2			Died
153	61	Ascorbic acid	16 mg.	11			No change
1850	25	Ascorbic acid	16 mg.	11			No change
7780	9	Ascorbic acid	16 mg.	11			Slight darkening after 10 weeks
191	14	Thyroid Adrenalin	1 grain 1.5 cc.	4	2 grains 1.5 cc.	10	Grey largely disappeared after 12 weeks, but reappeared on discontinuing the adrenalin

[1] Dog units.

after several failures a diet was found which will support growth in young guinea pigs, 200 to 275 gm. in weight and 3 to 5 weeks old. The diet contained extracted casein 30, sucrose or cornstarch 45.5, agar 5.0, salts 2.5, Crisco 2.0, and acetone extracted wheat germ 15.0 parts, with daily supplements of wheat germ oil 2 drops, ascorbic acid 8 mg., orange juice 3 cc., nicotinic acid 5 mg., and 0.25 cc. cod liver oil containing 0.04% carotene.

Four black guinea pigs of the same litter were placed on this diet in November, 1938, when their weights were 150 to 175 gm. All of them began to show dullness and metallic appearance of the fur in 6 to 8 weeks and one of them became completely grey by the last of January, 1939. The anti-grey factor was then given this animal but within 2 weeks it died still grey and in an emaciated condition. The fur of the other guinea pigs on the diet remained dull and metallic but did not grey and eventually all become cachectic and died. The guinea pig diet was not as deficient as that used for the rats because the wheat germ which it contained is by no means wholly free from the anti-grey factor.

Experiments with dogs and foxes

Eight young dogs were placed on a purified diet at 4 to 6 weeks of age with supplements of thiamin chloride, riboflavin and the wheat germ autolysate used for the rat experiments. Later crystalline synthetic vitamin B_6 [10] was used instead of the wheat germ preparation. Nicotinic acid was also given in some cases but not in all. The basal diet consisted of extracted casein 45.8, sucrose 20.9, cornstarch 19.4, Crisco 10.0, salts 2.4, and $CaCO_3$ 1.5 parts, and was supplemented by small amounts of carotene-reinforced cod liver oil.

Two black part-Boston bull female terriers were placed on this diet with nicotinic acid supplements, 3 mg. daily, late in January, 1939, at the age of 7 weeks when they weighed 1.7 kg. each. After 36 days their condition was serious, with

[10] We are indebted to Merck and Company for a supply of synthetic crystalline vitamin B_6.

frequent bloody diarrhea, stationary weight, and failure of appetite. Small doses of a filtrate concentrate were then given for 3 days with immediate improvement but after 30 days the symptoms recurred. The filtrate preparation was given for 4 days with immediate improvement again. After 40 days the weights again remained stationary.

After 2 to 4 months on the diet the intensely black fur of these dogs began to change to a reddish brown, first on the back of the neck then around the eyes and under the chin, and finally the fur was dull greyish over nearly the entire body surface. The teeth of both dogs rapidly discolored and pitted and the gums were somewhat reddish and swollen after 128 days on the diet. After 9 months on the deficient diet one of the dogs was given daily doses of the concentrated yeast filtrate preparation. After a few weeks repigmentation of the follicles and hair was clearly evident, the appetite had improved and the first estrus was established when the animal was 12 months of age. The other dog maintained on the deficient diet became steadily more emaciated, exhibited a bloody diarrhea and failed to show estrus, and was sacrificed in December, 1939, when moribund.

These and other dogs which received nicotinic acid but no filtrate preparation were affected more seriously by the deficiency than those which received neither of these factors.

Six young silver foxes at 2 months of age were placed on the diet used for the dogs, three receiving and three not receiving filtrate preparation, but all getting nicotinic acid and the other vitamin supplements. One fox on the deficiency diet died in 6 weeks and the other two grew a new coat of silvery white fur. Large mottled thymuses were found in the three animals on the deficient diet when sacrificed at 9 months of age, but no thymuses were evident in the three which received the filtrate preparation. A full account of this experiment will be published later. Survival of thymus tissue was detected also in some but not all of the rats which had greyed due to the deficiency.

The occurrence of unmistakable depigmentation in these young dogs and in one guinea pig as well as in several hundred rats and in two young silver foxes as a result of filtrate factor deficiency justifies the assumption that the greying phenomenon is truly a deficiency symptom. The concurrent skin and gland changes seen in the rats may well be suspected as being products of the same mechanism. These changes are typical of senescence, a process of obscure etiology. Perhaps the ageing process is conditioned by an increasing need for this sparsely distributed factor. In any case further study of the interrelation of the adrenal cortical and other hormones, the B vitamins and the phenomena of ageing appears justified.

SUMMARY

1. Young black or hooded rats were fed a basal diet adequate except with respect to the B vitamins and supplemented by thiamin chloride, riboflavin and a wheat germ preparation rich in vitamin B_6. The animals grew fairly well but within 6 to 10 weeks developed a greying of the fur, usually in bilateral patterns. Certain skin eruptions and in some cases persistent large skin ulcers appeared in many of the rats subsisting on this diet for several months.

2. Filtrates from fuller's earth-treated extracts of rice bran, yeast, liver, crude cane molasses and alfalfa in every case cured these symptoms but restored normal growth in varying degrees. Two filtrate factors appear to be involved, termed for convenience the anti-grey factor and the rat-growth filtrate factor.

3. Injection of commercial adrenal cortex and thyroid extracts cured the greying slowly but did not restore growth.

4. Histological study of adrenal glands, thyroids, skin and testes of these and similar rats deficient in vitamin B_6 or riboflavin showed that serious damage occurs in these tissues in the grey rats without corresponding changes in non-grey rats.

5. Lactation failed nearly completely in all rats placed on the filtrate factor-deficient diet on either the day of mating or the day of littering.

6. One guinea pig and eight young dogs have shown greying of the fur when subsisting on this deficient diet. The growth of the dogs was subnormal but the animals have survived for several months in spite of progressive greying of the hair, occasional diarrhea and poor appetite. Two young silver foxes were likewise affected.

7. The possible dependence of cortical adrenal and other gland function upon one of the members of the B complex and the relation of senescent changes to this deficiency are suggested.

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AVAILABILITY TO WHITE RATS OF PHOSPHORUS IN LESPEDEZA SERICEA AND ALFALFA HAYS

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THE PROBLEM

According to Thomas ('38) variations in phosphorus of 250% may occur within the same species of plant grown on different soil types. Orr ('29) discussed the variation in mineral content of pasture plants. Weathers ('38) presented data showing differences due to soil type on mineral content in certain hay crops in Tennessee. Auchter ('39) ably summarizes the new trend in agricultural thinking which emphasizes the interrelation of such factors as soils, plants, and animals, and efforts directed toward improving the nutritional quality of crops as well as increasing their yields. The aim of the present investigation is to compare the availabilty to the animal of the phosphorus of a low phosphorus hay and of a high phosphorous hay.

EXPERIMENTAL PROCEDURE FOR LESPEDEZA SERICEA

Rapidly-growing male white rats, 30 to 31 days old, and weighing from 43 to 79 gm., were used for a preliminary study of availability of phosphorus in hays.

Lespedeza sericea [1] was the type of hay studied in the first set of experiments. Two samples of stems, one of low and

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[1] The samples of lespedeza with analyses for minerals, nitrogen, and fiber were obtained from E. K. Weathers, Assistant General Chemist, U. T. Experiment Station.

one of high phosphorus content, and two samples of leaves of low and high phosphorus content were incorporated in four experimental diets. The rations as indicated in table 1 were so constructed that energy, fiber, mineral, and vitamin contents were identical for all animals in a given series, insofar as was possible, and all essential nutrients were present in adequate amounts with the exception of phosphorus. The amount of phosphorus for each diet was approximately 0.16% chosen on the basis of careful work done by Utley and MacLeod ('35) in this laboratory in constructing a diet which would supply either the minimal or slightly under the minimal amount of phosphorus for normal growth and phosphorus retention—an important consideration as emphasized by Mitchell and McClure ('37). In the control diet all of the phosphorus was supplied by salt mixture, whereas in the experimental diets 0.08% was supplied by salt mixture in each case and the remaining 0.08% by lespedeza. To make sure that animals could not be maintained in a normal nutritive condition on the 0.08% phosphorus supplied by the salt mixture without recourse to any phosphorus from lespedeza, four animals on the control diet containing 0.16% phosphorus as salt mixture were paired with four litter mates on a similar diet containing only 0.08% phosphorus as salt mixture. Food consumptions were kept uniform. To make the phosphorus content of all the experimental diets the same, different amounts of lespedeza were included for each diet. Calcium carbonate was added when necessary to supply 0.51% in each diet. This amount is adequate for normal growth and bone development. Thus the same Ca/P ratio of approximately 3 was maintained for all diets. To determine whether this Ca/P ratio was a disturbing factor in itself, animals on the control diet having a Ca/P ratio of 3 were paired with others on a control diet in which the calcium was reduced to give a Ca/P ratio of 1.5.

Comparisons were made by the paired feeding method between stems and leaves of low phosphorus content, 0.13% (0.29% P_2O_5) and 0.18% (0.41% P_2O_5) respectively, and between stems and leaves of high phosphorus content, 0.27%

(0.61% P_2O_5) and 0.26% (0.59% P_2O_5) respectively. A direct comparison was also made between the low phosphorus leaves and high phosphorus leaves. Since the phosphorus of feeds is often reported as phosphoric acid, the parenthetic values refer to the phosphorus in this form. Inclusion of control animals converted the paired to a triplicate feeding method. This method involved the use of three animals of the same sex, litter, and approximately the same weight, one animal being put on the control diet, and one each on the experimental diets being compared. The food consumption of the trio was kept the same by limiting two animals to the food intake of the one which ate the least. Thus, differences in growth and phosphorus retention due to varying food consumptions were ruled out. For the sake of comparison, some animals were allowed to eat each diet ad libitum.

The animals were started on the experimental diet at 30 to 31 days of age and killed at 60 to 61 days of age. The rats were ashed at approximately 550°C. and analyzed for phosphorus by the volumetric method essentially as approved by the Association of Official Agricultural Chemists ('35).

RESULTS FOR LESPEDEZA SERICEA

Table 2 summarizes the average results obtained for all animals. In both the direct and indirect comparison, rats on the diets containing low phosphorus lespedeza were less thrifty animals than their paired controls consuming the same amount of food whether judged on the basis of body weight, body length, or the amounts of phosphorus contained in the body. The animals on the diet containing the high phosphorus lespedeza much more nearly approached the control animals in regard to the various criteria used. Statistical analyses [2] (see table 2), using the phosphorus content of the body at 60 days of age as the criterion for comparison of the various diets, afford a mathematical interpretation of the data.

TABLE 1

Distribution of foodstuffs among the various constituents of the lespedeza and alfalfa diets[1]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	HAY IN DIET	PROTEIN FROM			CARBOHYDRATE FROM			FAT FROM BUTTER	FIBER FROM			CALCIUM FROM				PHOSPHORUS FROM			
DIETS		Hay	Lact.	Total	Hay	Starch[2]	Total		Hay	Agar	Total	Hay	Lact.	CaCO₃	Total	Hay	Lact.	H₃PO₄	Total
	%	%	%	%	approx. %	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Lespedeza Ca/P = 3																			
Control	0	0	18	18	0	40	40	8	0	28	28	0	0.036	0.476	0.512	0	0.025	0.125	0.150
Low P stems	63	5	13	18	28	10	38	8	28	0	28	0.435	0.026	0.050	0.511	0.080	0.018	0.067	0.165
Low P leaves	44	7	11	18	26	12	38	8	9	19	28	0.488	0.022	0	0.510	0.080	0.015	0.069	0.164
High P stems	30	3	15	18	13	26	39	8	13	15	28	0.198	0.030	0.280	0.508	0.080	0.021	0.062	0.163
High P leaves	31	6	12	18	17	21	38	8	6	22	28	0.369	0.024	0.120	0.513	0.080	0.016	0.068	0.164
Alfalfa Ca/P = 3																			
Control	0	0	18	18	0	59	59	8	0	9	9	0	0.036	0.449	0.485	0	0.027	0.128	0.155
Low P	37	7	11	18	19	38	57	8	9	0	9	0.477	0.022	0	0.499	0.079	0.017	0.070	0.166
High P	23	6	12	18	10	46	56	8	4	5	9	0.288	0.024	0.223	0.535	0.080	0.018	0.065	0.163
Alfalfa Ca/P = 5																			
Control	0	0	18	18	0	55	55	8	0	13	13	0	0.036	0.653	0.689	0	0.027	0.118	0.145
Low P	56	10	8	18	29	22	51	8	13	0	13	0.717	0.016	0	0.733	0.120	0.012	0.030	0.162
High P	35	8	10	18	15	36	51	8	8	5	13	0.371	0.020	0.348	0.739	0.123	0.015	0.024	0.162

[1] Columns 1, 3, 6, 8, 10, plus 4% salt mixture and 2% cod liver oil give the percentage compositions for each diet.

[2] Yeast extract was incorporated with the starch.

254

With diets as nearly identical as possible in nutritive quali-
fications and with phosphorus designed to be the limiting
factor for normal growth and bone development, it appears
that a difference exists in the availability of phosphorus in
the diet containing the lespedeza of low phosphorus content
both as compared to the control and to that containing the
lespedeza of high phosphorus content. That these differences
in availability of phosphorus were due to lack of absorption
and not to the type of phosphorus compound was indicated by
the amounts of feces obtained from animals on the different
diets (see table 2). In general the diets containing the higher
percentages of low phosphorus lespedeza produced the largest
amounts of feces, the diets containing smaller percentages of
high phosphorus lespedeza produced the next largest amounts
of feces, and the control diets with no lespedeza produced the
least. The cause for these differences in weights of feces is
not due to differences in bulk since the fiber contents of the
diets were equalized, but may be related to the relative indi-
gestibility of the nitrogen-free extracts of the diets containing
lespedeza. In view of the fact of large amounts of feces and
of definitely restricted growth and phosphorus retention of
animals on the diet containing lespedeza of low phosphorus
content, it seems possible that the large amount of hay neces-
sary to supply 0.08% phosphorus was not digested sufficiently
to make available for absorption the phosphorus contained
within the cellulose plant walls. Since the animals on the diets
containing lespedeza of high phosphorus content showed in-
creased growth and phosphorus retention, indicating that the
phosphorus of the lespedeza had been better utilized, it ap-
pears that the amounts of this high phosphorus lespedeza
necessary to supply the 0.08% phosphorus were small enough
to be well digested and assimilated.

Table 2 also compares the average amounts of phosphorus
from each diet stored by the rats during the experimental
period. Values for the amount of phosphorus in the food con-
sumed and the amount of phosphorus in the rat at the end of
the experiment were known by analysis. Using Sherman and

TABLE 2

Summary of average results for experimental animals in growth and phosphorus intake and retention including summaries from variance analyses for phosphorus content of rat at 60–61 days of age

EXPERIMENTAL GROUP	NO. OF CASES	WEIGHT Initial net	WEIGHT Final net	WEIGHT Gain net	LENGTH OF TRUNK	TOTAL FECES DRY WEIGHT	FOOD CONSUMPTION WITH A.D.[1]	TOTAL P IN FOOD WITH A.D.	P IN RAT AT 60–61 DAYS WITH A.D.	P IN RAT AT 30–31 DAYS WITH A.D.	P STORED FROM FOOD WITH A.D	PER CENT P FROM FOOD
		gm.	gm.	gm.	cm.	gm.	gm.	gm.	gm.	gm.	gm.	%
Triplicate feeding												
Low P lespedeza												
Control	5	53	136	83	16.8	111	293±18	0.440±0.027	0.728 ±0.040	0.281±0.053	0.448±0.020	102
Stems	5	54	92	38	14.8	199	294±16	0.485±0.027	0.528[a]±0.032	0.284±0.038	0.242±0.016	50
Leaves	5	53	90	37	15.0	201	295±16	0.483±0.026	0.554 ±0.020	0.281±0.036	0.273±0.028	57
F value for diets									184[a]			
Significant difference									0.028			
High P lespedeza												
Control	5	46	124	78	16.2	99	323±7	0.484±0.010	0.652±0.048	0.244±0.028	0.408±0.020	84
Stems	5	47	113	66	15.4	140	324±9	0.529±0.014	0.651±0.038	0.249±0.040	0.402±0.018	76
Leaves	5	46	104	57	14.9	161	324±9	0.531±0.015	0.590±0.041	0.245±0.037	0.345±0.030	65
F value for diets									257[a]			
Significant difference									0.023			
Significant difference for low vs. high P lespedeza									0.026			
Lespedeza leaves												
Control	4	56	115	58	15.1	85	256±48	0.384±0.072	0.510±0.107	0.296±0.009	0.214±0.075	77
Low P	4	54	74	20	13.4	151	259±46	0.424±0.076	0.464±0.080	0.286±0.016	0.178±0.064	42
High P	4	54	91	37	14.0	135	259±45	0.425±0.074	0.523±0.075	0.284±0.009	0.239±0.067	56
F value for diets									3.35[a]			
Significant difference									0.048			
Ad libitum feeding												
Control	2	68	181	113	18.0	150	455±19	0.683±0.028	0.932±0.031	0.361±0.022	0.572±0.010	84
Low P stems	1	72	132	60	15.8	234	405	0.668	0.738	0.382	0.356	53
Low P leaves	4	63	122	60	15.6	267	454±31	0.745±0.051	0.705±0.060	0.332±0.031	0.374±0.031	50
High P stems	1	56	166	110	17.8	198	454	0.740	0.920	0.297	0.623	84
High P leaves	5	67	161	94	17.0	247	490±18	0.803±0.030	0.908±0.089	0.357±0.014	0.549±0.026	68

Paired feeding												
Control Ca/P = 8	4	80	158	98	17.2	115[5]	349±10	0.524±0.024	0.798±0.089	0.818±0.029	0.475±0.062	91
Control Ca/P = 1.5	4	80	169	109	17.5	101[5]	349±17	0.559±0.026	0.886±0.013	0.317±0.028	0.569±0.016	102
F value for diets									9.85[4]			
Significant difference									0.093			
Control P = 0.16%	4	54	118	64	15.7	90	278±13	0.417±0.020	0.624±0.034	0.286±0.005	0.337±0.039	81
Control P = 0.08%	4	54	105	51	15.8	86	276±12	0.210±0.010	0.502±0.008	0.288±0.009	0.215±0.015	102
F value for diets									27.09[6]			
Significant difference									0.087			
Triplicate feeding												
Alfalfa Ca/P = 3												
Control	5	57	157	100	16.7	35	303±60	0.470±0.092	0.755±0.150	0.304±0.046	0.451±0.103	96
Low P	5	57	143	86	16.1	74	304±59	0.504±0.098	0.725±0.125	0.301±0.046	0.423±0.080	83
High P	5	57	145	88	16.2	62	303±60	0.493±0.099	0.752±0.130	0.302±0.043	0.450±0.087	91
F value for diets									8.15[3]			
Significant difference									0.037			
Alfalfa Ca/P = 5												
Control	5	54	110	56	15.9	34	222±40	0.322±0.058	0.584±0.055	0.288±0.015	0.296±0.056	92
Low P	5	54	88	34	14.7	78	223±39	0.361±0.063	0.523±0.054	0.286±0.011	0.237±0.054	66
High P	5	54	100	46	15.5	57	224±39	0.363±0.062	0.579±0.057	0.287±0.010	0.291±0.062	80
F value for diets									8.15[3]			
Significant difference									0.037			
Significant difference for Ca/P = 3 vs. Ca/P = 5									0.021			

[1] A.D. = average deviation.
[2] Average of four animals.
[3] F value exceeds the 1% point.
[4] F value does not exceed the 5% point.
[5] Average of two animals.
[6] F value exceeds the 5% point.

Quinn's ('26) average value of 0.53% phosphorus in the body
of rats at 28 days of age, the phosphorus present in the body
at the beginning of the experiment was estimated. From these
data the amount of phosphorus retained from the food during
the experimental period was calculated. In the series of diets
containing stems and leaves of lespedeza low in phosphorus,
the control animals retained all the phosphorus of the diet
whereas the experimental animals retained only about one-
half the amount supplied by the food. Considering that in the
experimental diets one-half of the phosphorus was supplied
by salt mixture and the other half by lespedeza, it seems prob-
able that here the rats had been able to use the phophorus
from the readily assimilable salt mixture but that most of the
phosphorus in the lespedeza was unavailable. In the series of
diets containing stems and leaves of lespedeza high in phos-
phorus, the estimations indicated that the control animals re-
tained, on the average, 84% of the phosphorus of the diet.
In the case of the experimental animals, we also find that both
those on stems and on leaves stored considerably more than
one-half the phosphorus of the food. In other words, in both
cases a considerable part of the phosphorus of the high phos-
phorus lespedeza was available to the animal. Similar rela-
tionships hold when the low and high phosphorus lespedeza
leaves were compared directly.

When animals on the diet containing lespedeza low in phos-
phorus content were allowed to eat ad libitum, their growth
and phosphorus retentions were improved over those of ani-
mals whose diets had been restricted by the triplicate feeding
method, but they did not equal those of animals allowed to eat
the control diet ad libitum. When animals on the diet of
lespedeza high in phosphorus content were allowed to eat ad
libitum, their growth and phosphorus retentions were not only
improved over those whose diets had been restricted but they
approached the ad libitum control animals in growth and
equaled them in phosphorus retention.

The results of the experiment to test the disadvantage re-
sulting from the disturbance in the Ca/P relationship, as

shown in table 2, gave slightly superior results in favor of the 1.5 to 1.0 ratio. Rates of growth, 158 and 169 gm., and phosphorus content of the body at 60 days, 0.793 and 0.886 gm., for Ca/P ratio of 3 and 1.5 to 1.0, respectively, were within normal limits as compared with those obtained by Sherman and Quinn ('26) for male rats at 61 days, 142 gm. and 0.814 gm. respectively. A variance analysis of these data indicates no difference of statistical significance between the two diets. Therefore, there could have been no serious disturbance in the present experiments as a result of an adverse Ca/P ratio.

At the termination of the experiment designed to show that 0.08% phosphorus is not sufficient for normal growth and phosphorus retention, the rats on the diet with 0.08% phosphorus contained, on the average, 0.502 gm. phosphorus in contrast to 0.624 gm. phosphorus for their paired litter mates consuming the same amount of food on the 0.16% level of phosphorus. Animals allowed to eat the diet containing 0.16% phosphorus ad libitum had, on the average, 0.932 gm. body phosphorus at the end of the experimental period. It is clear that 0.08% phosphorus in the diet is not enough to allow for normal growth and phosphorus retention.

The value of the paired feeding method is well illustrated in these experiments as a method of comparing two diets when the retention of a particular element, in this instance phosphorus, is the chief criterion for testing the diets rather than rate of growth. Table 3 indicates that, with no other change in the diet, both rate of growth and phosphorus content of the body increased with increased food consumption.

TABLE 3

Effect of food consumption on growth and phosphorus content of body

NUMBER OF CASES AVERAGED	FOOD CONSUMPTION	GAIN IN WEIGHT OVER 30-DAY EXPERIMENTAL PERIOD	P CONTENT AT END OF 30-DAY EXPERIMENTAL PERIOD
	gm.	*gm.*	*gm.*
2	208±12	46± 5	0.404±0.002
5	270± 5	74±12	0.648±0.056
15	323±17	91±10	0.704±0.069
2	455±19	123± 3	0.932±0.031

EXPERIMENTAL PROCEDURE FOR ALFALFA

It seemed desirable to test similarly samples of another type of leguminous hay varying in phosphorus content. Alfalfa was chosen because of the high place assigned to it as a feed. A preliminary difficulty arose in obtaining samples of alfalfa having as low a phosphorus content as that of the low phosphorus lespedeza. Out of five samples of alfalfa, two contained the high phosphorus content of 0.35% (0.80% P_2O_5) and one the relatively low phosphorus content of 0.21% (0.48% P_2O_5). The difference between the highest and lowest phosphorus contents is 0.14%, which is just about the difference between the high and low phosphorus contents of lespedeza used in the preceding experiments. The low phosphorus alfalfa, however, is almost as high as the high phosphorus lespedeza. Two sets of experiments were therefore initiated. In the first, the diets were constructed similarly to the lespedeza diets in that 0.08% phosphorus was supplied from alfalfa and 0.08% from a salt mixture. This meant that much smaller percentages of alfalfa were needed to supply the phosphorus than were required of the lespedeza. In the second set, 0.12% phosphorus was supplied by the alfalfa and only 0.04% by a salt mixture. This required amounts of alfalfa very similar to the amounts of lespedeza used. Since alfalfa is rich in calcium, the increased percentage of alfalfa in the diet increased the Ca/P ratio of 3 in the previous diets to one of 5. For the sake of brevity, the alfalfa diets deriving 0.08% of their phosphorus from alfalfa and 0.08% from salt mixture will be referred to as Ca/P = 3 diets, and those deriving 0.12% phosphorus from alfalfa and 0.04% from salt mixture will be referred to as Ca/P = 5 diets. The Ca/P ratio was kept the same for the three diets being compared in each series. No separation was made between stems and leaves, the comparisons being limited to that between an alfalfa high in phosphorus and one relatively low in phosphorus. The rest of the experimental procedure was similar to that followed with the lespedeza.

RESULTS FOR ALFALFA

For the series of Ca/P = 3 diets, reference to table 2 shows slight differences between the animals on the low phosphorus hay and either the controls or animals on the high phosphorus hay in respect to the various criteria used. These differences are too small, however, to be definitely considered significant although they approach significance. The difference between the animals on the low phosphorus hay and the controls is 0.030, whereas 0.037 is required for significance.

As in the lespedeza experiments, it is noted for the Ca/P = 5 diets that a high percentage of phosphorus from the food, 92% and 80%, was utilized by the controls and animals on the high phosphorus alfalfa, respectively, whereas only 66% was utilized by those on the low phosphorus alfalfa. This again correlates with the large amount of feces eliminated by the low phosphorus group and the smaller amounts for the high phosphorus and control groups.

In other words, it has been shown for alfalfa as well as for lespedeza that the phosphorus of a hay of low phosphorus content, necessitating the feeding of a large amount of hay to meet the phosphorus requirement for normal growth and bone development, is not so available to growing white rats as the phosphorus of the same variety of hay having a higher phosphorus content and therefore requiring a smaller amount to supply the phosphorus needs.

SUMMARY OF RESULTS

1. When the experimental diets contain phosphorus at a minimal level of adequacy and all other nutrients at optimal levels and the same for all diets, the phosphorus of a low phosphorus lespedeza sericea or alfalfa hay is less available to the rat for growth and bone development than the phosphorus of a high phosphorus hay of the same type.

2. A diet containing 0.16% phosphorus and a Ca/P ratio of 3 to 1 is just about minimal for normal growth and phosphorus retention when the diet is fed ad libitum or in slightly restricted amounts.

3. The paired feeding method eliminates the variable of different food consumptions.

CONCLUSION

On the basis of the above results, it would appear that rats consuming a relatively large percentage of hay in order to supply even the minimal amount of phosphorus, lose some of the nutrients, including phosphorus, through a lack of absorption due to increased elimination. With the phosphorus at the minimal level, it constitutes the limiting factor for growth and any appreciable loss of phosphorus results in symptoms of phosphorus deficiency. A hay having a higher phosphorus content and furnishing the minimal amount of phosphorus with a smaller amount of hay can apparently be retained in the intestines long enough for sufficient digestion to make the phosphorus available to the white rat.

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OBSERVATIONS ON THE AMOUNT OF ASCORBIC ACID REQUIRED TO MAINTAIN TISSUE SATURATION IN NORMAL ADULTS [1,2]

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Belser, Hauck and Storvick ('39) have reported a method for determining the minimum intake of ascorbic acid which will just maintain the tissues in a state of complete saturation as judged by the urinary excretion of ascorbic acid in response to various test doses.

In September, 1938, Dr. Hazel Hauck kindly sent us the details of the procedure being used in her investigation. This method has been used in our laboratory for three subjects and is reported here because it confirms the findings of Belser, Hauck and Storvick ('39) and also because data on blood plasma ascorbic acid were obtained which give additional information on the problem of tissue saturation and the possible requirement of vitamin C.

EXPERIMENTAL

Three college women who served as subjects lived on the basal diet and followed the procedure exactly as described by Belser, Hauck and Storvick ('39).

The titration method of Bessey and King ('33), modified to use 3% metaphosphoric acid as the extracting reagent, was used for the vitamin C determination of all the foods of the

basal diet except the beets and prunes. For these pigmented foods, the metaphosphoric acid extract was titrated by the method of McHenry and Graham ('35). Assuming that ascorbic acid was the only substance contributing to the reducing values as determined by titration, the basal diet, in the weighed amounts consumed daily, contained 20 mg. of ascorbic acid obtained as follows: pears and juice 2.8 mg., beets and juice 11.1 mg., evaporated milk 0.7 mg., carrots and juice 1.6 mg., prunes 2.8 mg., beef 1.0 mg. This basal diet contained approximately twice as much ascorbic acid as that reported by Belser and co-workers ('39); the beets accounted for the difference. Ascorbic acid supplements [3] were taken at breakfast time.

The experimental period lasted through March, April, and May, 1939. Twenty-four-hour samples of urine were collected in jars containing a preservative of sulphuric acid, hydroxyquinoline and toluene as recommended by Sendroy ('37). Urine preserved in this manner for 24 hours showed a loss of ascorbic acid of less than 5%.

The subjects were of the following height and weight, A.F.: 51.7 kg., 161.0 cm.; W.B.,: 51.3 kg., 162.0 cm.; N.T.: 66.7 kg., 173.5 cm.

Blood samples were taken in the morning before breakfast. About 0.8 cc. of blood was collected in a small vial from a finger prick and was analyzed immediately for ascorbic acid using the micro method of Farmer and Abt ('36).

RESULTS AND DISCUSSION

Urine. The response in urinary excretion to a 400 mg. test dose of ascorbic acid following a 4-day period when 200 mg. daily was taken, was determined for three successive periods for each subject. The daily excretion values are shown in table 1; not only were there differences in ascorbic acid excretion of individuals living on the same basal diet and with the same ascorbic acid intake, but there was also a definite

difference in response to the test dose by the same individual for the three periods. Subject A.F. also showed considerable variation in the day-to-day excretion. Both subjects A.F. and W.B. were found to have normal kidney function when tested for rate of phenolsulphonphthalein excretion.

TABLE 1

Blood plasma and urinary response to 400 mg. test dose of ascorbic acid following 200 mg. intake daily for 4 days by three human subjects

DAILY INTAKE OF ASCORBIC ACID IN ADDITION TO BASAL DIET		ASCORBIC ACID CONTENT					
		Urine: mg. per 24 hours			Blood:[1] mg. per 100 ml. plasma		
		A.F.	W.B.	N.T.	A.F.	W.B.	N.T.
Day	*mg.*						
First	200	139	...	210			
Second	200	133	167	177			
Third	200	105	167	187			
Fourth	200	131	190	185			
Fifth	400	170	281	277	1.70	1.84	1.48
Sixth	...				1.70	1.78	1.69
First	200	156	153	176			
Second	200	140	189	141			
Third	200	125	179	136			
Fourth	200	98	183	161			
Fifth	400	285	272	327	1.63	1.34	1.31
Sixth	...				1.55	1.29	1.42
First	200	135	184	193			
Second	200	121	191	156			
Third	200	148	168	166			
Fourth	200	111	...	163			
Fifth	400	270	229	308	1.52	1.30	1.42
Sixth	...				1.33	1.32	1.40

[1] Blood plasma determinations were made on the morning of taking 400 mg. ascorbic acid and on the following morning.

The lowest level of ascorbic acid which was excreted in response to the 400 mg. test dose was taken as the criterion for complete saturation for each subject and was as follows: subject A.F., 170 mg.; subject W.B., 229 mg.; subject N.T., 277 mg.

Time did not permit investigation of as many levels of intake as Hauck and co-workers used; nevertheless, sufficient

data were obtained to indicate the probable requirement of each subject in order to maintain tissue saturation under the specified conditions. These data are summarized in table 2. Subject A.F. showed tissue saturation as judged by the 400 mg. response following an intake of 70 mg. daily for 6 days. Daily excretions of this subject were variable and a lower response was obtained after 100 mg. daily intake than when receiving 90 mg. daily. A similar variation in response has been noted by Belser, Hauck and Storvick ('39) with some of their subjects. Subject W.B. attained tissue saturation following a daily intake of 60 mg.; at the 70 mg. level of intake there was practically no increase in the 400 mg. response and when the test at this level was repeated the 400 mg. response was lowered. This further confirmed our previous observation that there is a wide range in excretion of ascorbic acid for the same individual under the same conditions of ascorbic acid intake. Subject N.T. gave approximately the required response to the 400 mg. test dose following a 6-day intake of 90 mg. of ascorbic acid daily.

From the data in table 2, it was concluded that, in addition to the basal diet, subject A.F. required approximately 70 mg., subject W.B. 60 mg., and subject N.T. more than 90 mg. to maintain tissue saturation.

When the ascorbic acid intake of 20 mg. daily from the basal diet was added to these values and the requirement then calculated on the basis of body weight the following were the requirements per kilogram of body weight: A.F., 1.7 mg., W.B., 1.6 mg. and N.T., more than 1.6 mg. Belser and co-workers ('39) reported that from 1.0 to 1.6 mg. per kilogram was required by their subjects.

Blood. It is possible that the blood plasma levels of ascorbic acid are a more reliable index of the state of ascorbic acid nutrition than the urine levels. There are many sources of error in the urinary determination and little is known of the amount or rate of destruction of ascorbic acid in its passage through the kidneys and ureters and while retained in the bladder.

TABLE 2

Blood plasma and urinary response to 400 mg. test dose following different levels of ascorbic acid intake [1] for 6-day periods

SUBJECT A.F.			SUBJECT W.B.			SUBJECT N.T.		
	Ascorbic acid			Ascorbic acid			Ascorbic acid	
Intake	Urine	Plasma	Intake	Urine	Plasma	Intake	Urine	Plasma
mg.	*mg. per 24 hr.*	*mg. per 100 ml*	*mg.*	*mg. per 24 hr.*	*mg. per 100 ml.*	*mg.*	*mg. per 24 hr.*	*mg. per 100 ml.*
40 [2]	21	1.68	60	90	1.48	40	27	1.47
40 [2]	22	....	60	79	1.41	40	16	....
40 [2]	18	....	60	70	1.34	40	22	....
40 [2]	5	....	60	39	1.14	40	21	1.16
40 [2]	15	....	60	32	1.16	40	14	1.28
40 [2]	8	....	60	33	....	40	19	1.08
400	153	1.17	400	242	1.12	400	130	1.10
		1.67 [3]			1.61 [3]			1.64 [3]
70	62	1.50	70	70	1.42	80	114	1.50
70	29	1.36	70	63	1.29	80	72	....
70	12	1.28	70	58	1.18	80	85	1.37
70	26	1.04	70	64	1.04	80	68	1.29
70	33	....	70	57	....	80	54	1.14
70	16	1.06	70	47	1.12	80	50	1.16
400	175	1.06	400	250	1.12	400	245	1.19
		1.48 [3]			1.66 [3]			1.60 [3]
90	44	1.36	70	95	1.52	90	110	1.41
90	46	....	70	76	....	90	82	1.23
90	24	1.34	70	71	1.28	90	83	....
90	40	1.26	70	50	1.26	90	83	1.11
90	38	1.19	70	46	1.14	90	71	1.03
90	68	1.21	70	43	1.10	90	69	....
400	280	1.12	400	210	1.14	400	263	0.99
		1.41 [3]			1.58 [3]			1.56 [3]
100	120	1.60						
100	25	....						
100	102	1.33						
100	53	1.31						
100	28	1.26						
100	31	1.06						
400	248	1.04						
		1.43 [3]						

[1] The intake noted was taken as crystalline ascorbic acid in addition to the basal diet.

[2] Ascorbic acid was taken as red raspberries calculated to contain 40 mg. ascorbic acid.

[3] Blood plasma values 24 hours after ingestion of 400 mg. dose.

In the first part of the study, blood determinations were made in the morning when the 400 mg. test dose was given, and on the following morning; i.e., at the end of the 24-hour urinary collection period. The data are summarized in table 1; for each subject there was practically no difference in blood level before and after taking the 400 mg. test dose. It was concluded that the subjects were saturated before taking the test dose since the 400 mg. test dose did not raise the blood level of ascorbic acid; during the 24-hour excretion period the excess ascorbic acid was removed from the blood, either being oxidized to some other form or excreted in the urine. The plasma levels for these saturated subjects for the three periods ranged from 1.33 to 1.70 for A.F., from 1.29 to 1.84 for W.B., and from 1.31 to 1.69 for N.T. These values are in agreement with the reports of Faulkner and Taylor ('38) and others (Wright, '38; Lund, '37; Goldsmith and Ellinger, '39) that the renal threshold for ascorbic acid is approximately 1.4 mg. per 100 ml. of blood plasma.

In the second phase of the investigation, daily determinations of the blood plasma ascorbic acid were made for all subjects, except for subject A.F. when on the 40 mg. intake level. The individual daily blood values are given in table 2 and represent the plasma content of ascorbic acid on the morning of each day of ascorbic acid intake and also on the morning following the 400 mg. test dose. The blood values at the beginning of each period ranged from 1.36 to 1.68 mg. for all subjects and indicated saturation; the blood level then fell during the 6-day period for each subject but was immediately raised to 1.4 mg. or above on the morning following the 400 mg. test dose.

Faulkner and Taylor ('38) reported that a maintained value of blood ascorbic acid at or above the threshold value corresponds to saturation, and below the threshold level corresponds to unsaturation. However, it is still an open question whether the blood level should be maintained at 1.4 mg. for optimum health; more than 120 mg. daily was required by each

subject to maintain this level. Wright ('38) has stated that the normal blood plasma level of ascorbic acid in adults lies between 0.7 and 1.3 mg. per 100 ml. The data in table 2 show that both subjects A.F. and N.T. had a blood level above 0.7 mg. when the total daily intake was 60 mg. (basal diet plus 40 mg.) of ascorbic acid, the values being 1.17 mg. and 1.10 mg. respectively.

If a blood plasma level of 0.7 mg. and an increase to 1.4 mg., 24 hours after taking a 400 mg. test dose is accepted as a measure of tissue saturation, then 60 mg. of ascorbic acid daily was above the requirement of these subjects. If a blood value maintained at approximately 1.4 mg. is taken as saturation then 120 mg. daily was inadequate.

SUMMARY

Using a method for estimating the minimum intake of ascorbic acid required to maintain the tissues in a state of complete saturation as judged by urinary excretion of ascorbic acid, the requirement for three adult subjects was found to be from 1.6 to 1.7 mg. per kilogram per day. The urinary excretion was found to vary considerably for the same individual even under carefully controlled conditions of ascorbic acid intake.

Values above 1 mg. of ascorbic acid per 100 ml. of blood plasma were obtained when the total daily intake was 60 mg.; more than 120 mg. daily were required to raise the blood plasma to 1.4 mg. The significance of the blood plasma levels in relation to saturation is discussed.

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THE INDEPENDENCE OF THE ENDOGENOUS AND THE EXOGENOUS METABOLISM OF NITROGEN [1]

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The theory of protein metabolism proposed by Folin ('05) distinguishes between two kinds of protein catabolism:

One kind is extremely variable in quantity, the other tends to remain constant. The one kind yields chiefly urea and inorganic sulphates, no kreatinin, and probably no neutral sulphur. The other, the constant katabolism, is largely represented by kreatinin and neutral sulphur, and to a less extent by uric acid and ethereal sulphates. The more the total katabolism is reduced, the more prominent become these representatives of the constant katabolism, the less prominent become the two chief representatives of the variable katabolism.

The constant type of metabolism Folin called the endogenous metabolism and because of its constancy and its independence of dietary protein, he referred it to the tissues of the body and assumed that it represented ''an essential part of the activity which distinguishes living cells from dead ones.'' The variable protein metabolism was called the exogenous metabolism and was referred to the disposition of dietary protein.

Although the theory of Folin has dominated later investigations of this subject and has been confirmed in many of its details, no one of its basic theses has escaped at least an occasional denial, because of evidence that seems impossible to reconcile with it.

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[1] The substance of this paper was taken from a thesis submitted by E. Wise Burroughs to the Graduate School of the University of Illinois in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Animal Husbandry, July, 1939.

The independence of the endogenous and exogenous types of metabolism has been denied on the basis of indications that certain amino acids incorporated in the diet may either stimulate or depress the endogenous metabolism. Such indications, to be valid, should be obtained with animals that have been so prepared that the urinary nitrogen is all of endogenous origin, including no nitrogen of immediate dietary origin nor any originating from the catabolism of the labile stores of protein in the body, sometimes referred to as "deposit protein." They should also be sufficiently definite and reproducible so that they can be clearly distinguished from the normal variations in the output of endogenous nitrogen.

The most serious challenge to the independence of the endogenous and exogenous types of protein (nitrogen) metabolism is provided by the recent investigations of Schoenheimer and associates, using isotopic nitrogen to label the amino acids whose metabolism was studied. These investigations have revealed a rapid and extensive interchange of nitrogen between dietary amino acids and tissue proteins that presumably results from chemical reactions involving the opening of peptide linkages, deamination, reamination or even transamination of amino acid residues, with re-entrance into positions in the tissue protein molecules left vacant by the rupture of peptide linkages. In their most recent publication, Schoenheimer and his colleagues (Schoenheimer, Ratner and Rittenberg, '39) conclude: "It is scarcely possible to reconcile our findings with any theory which requires a distinction between these two types of nitrogen," referring to endogenous and exogenous nitrogen.

However, it may reasonably be questioned whether these ingenious and revealing investigations of Schoenheimer bear any relation at all to the endogenous metabolism of Folin. Mature, or nearly mature, rats subsisting upon diets containing 16 to 18% of protein must have very considerable stores of proteins of the labile type ("deposit protein"), that are depleted and repleted readily with every fall and rise of the intake of dietary protein. Whipple ('38) speaks of the "fluid-

ity'' of these reserve stores, which he has shown in his own investigations to be readily available for the regeneration of hemoglobin and plasma protein after depletion by surgical hemorrhage and plasmapheresis, respectively. He distinguishes clearly, as Folin would, between these dispensable reserve proteins and the indispensable fixed proteins of the tissues. In all likelihood, the chemical reactions that Schoenheimer has detected by means of isotopic nitrogen between dietary amino acids and tissue proteins relate not to the fixed proteins of the cells, indispensable to their normal functioning, but to the dispensable reserve proteins, readily subject to mobilization by many experimental procedures and as readily reformed. In any studies of the endogenous metabolism, these protein stores must be reduced to zero by the continued feeding of diets containing only inconsiderable amounts of nitrogen.

The losses of nitrogen incurred in the endogenous catabolism presumably determine the minimum amounts of nitrogen required for the maintenance of life. The fact that these losses may be partially replaced by incomplete proteins, and simple amino acid mixtures, or even by individual amino acids, would seem to imply that the losses in the main do not represent the disintegration of tissue proteins, but of some nitrogenous constituents of the tissues of much simpler structure than proteins. On the other hand, Nielsen, Gerber and Corley ('39) and Nielsen and Corley ('39) have concluded that the diminished losses of body nitrogen brought about by supplementing a nitrogen-free diet with individual amino acids or simple amino acid mixtures, may be interpreted either as a retention (utilization) of part of their contained nitrogen, or as a sparing of an equivalent amount of nitrogen from the tissues. The latter interpretation involves a depression of the endogenous catabolism, distinctly at variance with Folin's theory.

The choice between these two interpretations seems a difficult one, although the fact that the creatinine excretions were stated to be constant is presumptive evidence that the endogenous nitrogen metabolism had not been depressed. Other evidence of the same sort that the endogenous metabolism is

neither depressed nor stimulated by dietary sources of nitrogen is afforded by the experiments of Terroine and Sorg-Matter ('27, '28) and of Smuts ('35) in which the endogenous metabolism is shown to be related closely to the basal metabolism of energy. Also, Mitchell, Burroughs and Beadles ('36) have concluded that the assumptions underlying the calculation of the biological values of proteins by the method developed in this laboratory, including the assumption of the independence of the endogenous and the exogenous metabolism of nitrogen, are essentially correct, since the values thus obtained are confirmed by an independent method.

The purpose of the investigations to be reported in this and two later papers was to study the endogenous metabolism of nitrogen by the use mainly of pure amino acids and mixtures of pure amino acids as the sole considerable source of dietary nitrogen. In this paper the experiments reported relate to the possibility of depressing the endogenous metabolism by the ingestion of small amounts of individual amino acids, of simple mixtures of amino acids, or of a protein mixture (egg) of high biological value. If the urinary nitrogen excreted on a nitrogen-free diet represents the catabolism of the amino acids of tissue origin left over after certain specific requirements for hormone-precursors have been met, according to an opinion not infrequently expressed, then the feeding of amino acids that may serve such purposes, in small amounts, would be expected to result in a sparing of tissue protein and consequently in a disproportionately large reduction in the excretion of nitrogen in the urine.

PLAN AND METHODS

Albino rats of both sexes and of mature or approximately mature weight were used as subjects of the experiment. The plan of the experiment involved three consecutive experimental periods of 2 to 5 days each (generally 3 or 5) following a preliminary period of 5 or 6 days' duration. Throughout this time the rat received a nitrogen-free diet, supplemented

daily by 20 mg. of yeast concentrate [2] to provide the vitamins of the old B complex. During the second experimental period the rats received daily, separate from the basal ration, a small supplement of a single amino acid, a simple mixture of amino acids, or egg protein. The effect of the nitrogen supplement on the endogenous catabolism was assessed by comparing the excretion of urinary nitrogen of the second period with the average excretion of the first and third periods.

The nitrogen-free diet contained 63% starch, 10% sucrose, 12% lard, 8% butterfat, 5% salt mixture (modified from Osborne and Mendel to include cobalt, copper, and zinc), and 2% cod liver oil. An attempt was made to maintain constant the intake of food for each rat throughout its three experimental periods.

The rats were kept in individual metabolism cages and the collections of urine and feces were made according to the procedure used in this laboratory in the determination of the biological value of proteins. For a description of cages and procedure the article by Smuts ('35) may be consulted.

The feces of the different experimental periods were separated sharply by the use of markers, ferric oxide and chromic oxide being used alternately. The urine was completely removed from the urinary bladder daily, either by catheterization in the case of female rats, or by pressure applied over the ureters. With training, the animals could be induced to respond to the latter method as satisfactorily (as tested by the catheter) as to the former, and with much less time and trouble on the part of the operator.

The urine was preserved with sulfuric acid and toluene. Both feces and urine were analyzed for total nitrogen by the Kjeldahl-Gunning-Arnold method, and the urine was analyzed colorimetrically for creatinine, using a photo-electric colorimeter and a purified creatinine standard.

The validity of the method of detecting a possible effect of the nitrogenous supplements upon the endogenous metabolism

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[2] From the Harris Laboratories, Tuckahoe, N. Y.

by comparing the urinary nitrogen of the second experimental period with the average nitrogen excretion of the first and third periods of nitrogen-free feeding, depends either upon a constancy of the output of endogenous nitrogen or upon a rectilinear change from period 1 to period 3. Preliminary tests upon three adult rats indicated that either condition may exist after a preliminary feeding period of 5 days.

TABLE 1

Average daily excretion of urinary nitrogen in the first and third periods of nitrogen-free feeding compared with the second period

RAT NO.	BODY WEIGHT	LENGTH OF EACH PERIOD	FIRST PERIOD	THIRD PERIOD	AVERAGE FIRST AND THIRD	SECOND PERIOD	DIFFERENCE
	gm.	*days*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	261	3	58	41	50	48	—2
2	236	3	55	45	50	51	1
3	236	3	57	42	50	45	—5
1	258	3	48	36	42	41	—1
2	230	3	51	40	45	45	0
3	232	3	45	36	40	42	2
1	255	3	41	33	37	36	—1
2	224	3	45	37	41	40	—1
3	227	3	42	35	38	36	—2
6	349	5	98	62	80	80	0
10	406	5	73	59	66	67	1
16	285	5	66	50	58	59	1
6	356	3	103	80	92	89	—3
10	410	3	76	66	71	69	—2
16	291	3	68	59	63	63	0
6	347	3	89	66	78	80	2
10	405	3	69	60	65	66	1
16	284	3	63	50	57	59	2
Average			64	50	57	57	0

To put the question to a quantitative test, six rats were fed the nitrogen-free diet for a period of 20 days. After the first 5 days, which were considered a preliminary adjustment period, urine collections were made for successive 3- or 5-day periods. Then for each rat, the average daily excretions of urinary nitrogen for series of three successive periods were studied by comparing the excretion of the middle period with the average excretion of the initial and final periods. The results of this study are summarized in table 1.

The results indicate that during the second period of specific nitrogen inanition the excretion of urinary nitrogen was approximately midway between that of the first and third periods, the positive and negative deviations from mid-position averaging approximately zero. The standard deviation of the differences between the excretions of the second period and the average excretions of the first and third periods is 1.88 mg. of nitrogen. From the values of the normal probability integral (Davenport and Ekas, '36), it may be computed that the deviations from zero corresponding to probabilities of a fortuitous event of 0.01, 0.02, and 0.03 are 4.38, 3.85, and 3.53 mg. of urinary nitrogen. This may be taken to mean that if in a middle test period, in which an amino acid supplement is fed, the daily urinary excretion of nitrogen deviates from the average daily excretion of the first and third periods of nitrogen-free feeding by 4 mg. or more, the deviation may be considered to be statistically significant. This implies, of course, that the endogenous metabolism pursues a constant or a rectilinearly changing course throughout the three experimental periods.

DISCUSSION OF EXPERIMENTAL RESULTS

The essential results of the experiment are collected in tables 2, 3 and 4. In tables 2 and 3, a positive difference (last column of tables) in the excretion of urinary nitrogen between the test period and the initial and final periods of nitrogen-free feeding would, if statistically significant, testify to the incomplete utilization of the nitrogenous supplement tested. Although the results for the various supplements used are not entirely concordant, there is evidence of incomplete utilization, or complete failure of utilization, in the case of tryptophane, phenylalanine, leucine, and cystine.

A negative difference, representing a lower excretion of urinary nitrogen in the middle test period than in the initial and final periods of complete nitrogen inanition, would represent, if statistically significant, a depression of the endogenous metabolism. Among the forty-three experiments on thirteen individual amino acids reported in table 2, only thirteen gave

TABLE 2

The influence of small amounts of individual amino acids on the endogenous metabolism of nitrogen

AMINO ACID TESTED	RAT NO.	AVERAGE BODY WEIGHT	DAILY N INTAKE IN 2ND PERIOD	DAILY URINARY NITROGEN EXCRETION				
				Initial N-free period	Final N-free period	Average of N-free periods	Amino acid period	Difference
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Threonine	10	191	4.7	41.9	34.4	38.2	39.7	+1.5
Threonine	11	226	4.7	55.3	38.9	47.1	49.9	+2.8
Threonine	12	231	4.7	50.7	40.3	45.5	47.9	+2.4
Isoleucine	10	191	4.3	34.4	38.3	36.4	36.6	+0.2
Isoleucine	11	226	4.3	38.9	42.3	40.6	41.2	+0.6
Isoleucine	12	231	4.3	40.3	41.2	40.8	41.5	+0.7
Tryptophane	5	357	7.0	74.7	58.3	66.5	76.8	+10.3
Tryptophane	7	379	7.0	80.6	57.1	68.8	72.9	+4.1
Valine	27	321	4.8	85.3	59.8	72.6	72.7	+0.1
Valine	36	279	4.8	67.5	53.7	60.6	63.2	+2.6
Valine	37	263	4.8	53.9	39.8	46.8	50.3	+3.5
Methionine	15	330	5.6	77.1	39.4	58.2	40.4	−17.8
Methionine	27	345	4.0	83.1	57.6	70.4	63.3	−7.1
Methionine	28	323	4.8	83.6	52.6	68.1	63.5	−4.6
Methionine	4	221	4.7	46.5	33.3	39.9	37.1	−2.8
Methionine	5	217	4.7	42.1	25.7	33.9	41.7	+7.8
Methionine	6	218	4.7	36.0	32.3	34.2	35.1	+0.9
Lysine	7	216	3.8	50.3	38.0	44.2	46.1	+1.9
Lysine	8	195	3.8	47.0	39.4	43.2	43.7	+0.5
Lysine	9	216	3.8	43.3	37.4	40.3	42.3	+2.0
Histidine	30	354	5.5	67.8	46.8	57.3	56.0	−1.3
Histidine	31	351	5.5	76.9	59.8	68.4	67.2	−1.2
Histidine	32	374	5.5	90.8	61.9	76.4	72.6	−3.8
Phenylalanine	33	399	6.0	79.3	51.4	65.4	69.6	+4.2
Phenylalanine	34	353	6.0	79.7	59.9	69.8	65.6	−4.2
Phenylalanine	35	308	6.0	76.8	71.8	74.3	80.2	+5.9
Leucine	19	347	6.0	74.6	56.9	65.8	69.3	+3.5
Leucine	29	361	6.0	99.0	78.4	88.7	101.4	+12.7
Leucine	36	351	6.0	80.8	54.1	67.4	77.2	+9.8
Arginine	4	227	6.0	47.7	46.5	47.1	45.9	−1.2
Arginine	5	221	6.0	48.1	42.1	45.1	41.2	−3.9
Arginine	6	222	6.0	40.7	36.0	38.4	40.9	+2.5
Cystine	11	322	4.7	64.1	61.6	62.9	64.3	+1.4
Cystine	12	329	4.7	64.1	53.7	58.9	56.2	−2.7
Cystine	13	336	4.7	73.9	53.9	63.9	55.0	−8.9
Cystine	11	308	11.4	61.6	48.7	55.1	56.6	+1.5
Cystine	12	318	11.4	53.7	50.4	52.0	61.2	+9.2
Cystine	13	325	10.6	53.9	47.6	50.8	55.5	+4.7
Tyrosine	12	349	6.0	68.9	65.3	67.1	70.7	+3.6
Tyrosine	13	396	6.0	103.6	74.0	88.8	79.6	−9.2
Glutamic acid	7	216	5.7	38.0	36.5	37.2	39.1	+1.9
Glutamic acid	8	195	5.7	39.4	32.7	36.0	36.3	+0.3
Glutamic acid	9	215	5.7	37.4	35.1	36.2	40.8	+4.6

negative differences and of these differences, only six exceeded in magnitude the 4 mg. previously taken as the limiting value between statistical insignificance and statistical significance. These six cases relate to methionine (three cases), phenylalanine (one case), cystine (one case), and tyrosine (one case). They were all obtained in experiments in which the urinary nitrogen in the first nitrogen-free feeding period was unusually high, and greatly in excess of the nitrogen excretion

TABLE 3

The influence of small amounts of simple mixtures of amino acids and of egg proteins on the endogenous metabolism of nitrogen

AMINO ACID MIXTURE OR PROTEIN TESTED	RAT NO.	AVERAGE BODY WEIGHT	DAILY N INTAKE IN 2ND PERIOD	DAILY URINARY NITROGEN EXCRETION				Differ-ence
				Initial N-free period	Final N-free period	Average of N-free periods	Amino acid or protein period	
		gm.	mg.	mg.	mg.	mg.	mg.	mg.
Mixture no. 1[1]	1	236	4.11	58.3	44.7	51.5	43.1	—8 4
Mixture no. 1	2	235	4.11	58.7	35.5	47.1	37.0	—10.1
Mixture no. 1	3	233	4.11	58.3	42.6	50.5	41.8	—8.7
Mixture no. 1	1	222	4.11	41.7	37.2	39.4	43.1	+3.7
Mixture no. 1	2	230	4.11	33.0	32.9	32.9	31.9	—1.0
Mixture no. 1	3	223	4.11	37.0	31.4	34.2	36.8	+2.6
Mixture no. 2[2]	4	227	4.76	54.5	35.7	45.1	43.1	—2.0
Mixture no. 2	5	216	4.76	61.7	43.3	52.5	45.3	—7.2
Mixture no. 2	6	217	4.76	54.2	36.5	45.4	42.7	—2.7
Mixture no. 2	4	213	4.76	35.0	35.0	35.0	40.4	+5.4
Mixture no. 2	5	203	4.76	44.4	43.5	44.0	43.6	—0.4
Mixture no. 2	6	206	4.76	33.7	34.1	33.9	41.1	+7.2
Mixture no. 3[3]	7	201	4.44	53.9	35.6	44.8	35.0	—9.8
Mixture no. 3	8	213	4.44	52.5	29.0	40.8	34.0	—6.8
Mixture no. 3	9	192	4.44	60.1	31.7	45.9	38.0	—7.9
Mixture no. 3	7	190	4.44	34.3	31.3	32.8	33.2	+0.4
Mixture no. 3	8	204	4.44	28.2	29.1	28.6	28.8	+0.2
Mixture no. 3	9	182	4.44	33.1	30.0	31.5	29.7	—1.8
Mixture no. 4[4]	72	168	8.0	29.4	26.5	28.0	30.6	+2.6
Mixture no. 4	75	177	8.0	25.6	24.2	24.9	26.4	+1.5
Egg proteins	39	218	22.4	36.4	32.5	34.4	33.9	—0.5
Egg proteins	40	220	22.4	38.6	32.7	35.6	36.2	+0.2

[1] This mixture contained equal quantities of nitrogen from methionine, histidine, arginine, isoleucine and lysine.

[2] This mixture contained equal quantities of nitrogen from valine, threonine, phenylalanine, tryptophane and leucine.

[3] This mixture is a combination of mixtures 1 and 2 in equal proportions.

[4] The nitrogen of this mixture was distributed as follows: threonine 15, isoleucine 15, valine 12, methionine 12, tyrosine 10, tryptophane 6, norleucine 30.

in the final period of nitrogen-free feeding. Furthermore, in none of the six cases suggesting a depression of the endog-enous metabolism was the nitrogen excretion of the amino acid period less than that of the final period of nitrogen-free feeding. These facts indicate that the decrease in the rate of the endogenous metabolism was unusually steep and quite possibly was not rectilinear in shape. Also, for each of these four amino acids, other experiments in which generally better agreement between the excretions of the first and third periods were obtained, three in the case of methionine, two in the case of phenylalanine, five in the case of cystine, and one in the case of tyrosine, gave no evidence of a depression of the endogenous metabolism.

Of the twenty-two experiments reported in table 3, nine gave positive differences in urinary nitrogen excretion between the test period, during which a supplement of an amino acid mixture or a complete protein (egg) was given, and the average excretion for the initial and final periods on the basal nitrogen-free diet, although only two were of sufficient magnitude to indicate incomplete utilization. Of the thirteen negative differences, seven exceeded 4 mg., but all were ob-tained in experiments in which the indicated decrease in endog-enous metabolism from the first to the third experimental periods was unusually great, ranging from 13.6 to 28.4 mg. of nitrogen daily. Also, for the first three amino-acid mixtures for which these large negative differences were obtained, at least three other experiments in each case, in which more favorable conditions for detecting a depression of the endog-enous nitrogen prevailed, because of nearly equal urinary nitrogen excretions in periods 1 and 3, revealed no evidence of such a depression. These experiments are repetitions on the same rats of experiments yielding the suspiciously large negative differences.

Thus, the evidence as a whole does not support the proposi-tion that the endogenous metabolism of nitrogen may be depressed by the feeding of single amino acids, of amino acid mixtures, one of which (no. 4) has been shown to support

nitrogen equilibrium in the rat, or of a complete protein mixture (egg) of proved high biological value.

Creatinine determinations were made on the urine collections of a number of these experiments. The average amounts of creatinine nitrogen in the urine daily, as well as the percentages of creatinine nitrogen on the total urinary nitrogen, for all rats receiving the same amino acid supplement and for all experimental periods, are summarized in table 4.

· These results are of interest in showing a fairly consistent increase in the percentage of creatinine nitrogen in the urine

TABLE 4

Average daily excretion of creatinine nitrogen for the three experimental periods for each nitrogenous supplement used

AMINO ACID	INITIAL N FREE PERIOD		AMINO ACID PERIOD		FINAL N-FREE PERIOD	
	Total	Urinary N	Total	Urinary N	Total	Urinary N
	mg.	*%*	*mg.*	*%*	*mg.*	*%*
Threonine	2.95	6.02	3.16	6.94	1.60	4.24
Isoleucine	1.60	4.24	3.09	7.80	2.86	7.03
Valine	4.06	6.00	3.96	6.46	3.87	7.72
Histidine	5.80	7.40	6.24	9.50	6.03	10.68
Lysine	3.28	7.05	2.95	6.73	2.86	7.49
Phenylalanine	6.01	7.64	6.41	9.02	4.46	7.10
Cystine	3.23	5.29	3.25	5.59	3.35	6.41
Glutamic acid	2.86	7.49	3.05	7.86	2.81	8.02
Mixture no. 1	2.63	5.80	2.68	7.01	2.60	7.06
Mixture no. 2	3.20	7.08	3.18	7.45	2.99	7.93
Mixture no. 3	2.73	6.67	2.72	8.27	2.58	8.31
Average	3.49	6.40	3.70	7.51	3.27	7.45

from period 1 to period 2, suggesting that all of the deposit protein had not been catabolized in the preliminary period. The general decrease in observed daily urinary nitrogen from period 1 to period 3 may thus be largely accounted for in this way. With respect to the average amounts of creatinine nitrogen excreted daily in the various periods, there were no consistent differences, although in the averages for all rats, the excretion in period 2 exceeded those in the adjacent periods. For the various amino acids there was no consistent indication that the creatinine excretion was increased by amino acid

feeding, although it should be remembered that only small amounts were fed.

An attempt was made to maintain the food intakes of the rats constant in all three experimental periods, and generally the attempt was successful. In all experiments reported in table 3, the daily intake of basal ration was identical in the three periods. In twenty-three of the forty-three experiments reported in table 2, this ideal was also realized, and in twelve other experiments it was approximately realized. Since the excretion of endogenous nitrogen is apparently not affected by variable intakes of a nitrogen-free diet unless the intake of energy is distinctly inadequate (Mitchell, '24), it is not believed that the failures to maintain a constant intake of food throughout these experiments seriously modified the excretion of urinary nitrogen.

The excretions of nitrogen in the feces, although measured in all of these experiments, will not be reported, because they indicate complete absorption of the supplementary nitrogen fed. The average excretions of fecal nitrogen daily per gram of food consumed for all experiments were 1.63 mg. in period 1, 1.53 mg. in period 2, and 1.46 mg. in period 3.

SUMMARY AND CONCLUSIONS

Although thirteen individual amino acids, including all of those considered essential for growth, four amino-acid mixtures, including one that proved adequate for the maintenance of nitrogen equilibrium in the adult rat, and one complete protein mixture of high biological value (egg) were tested, no evidence was obtained that the endogenous metabolism could be depressed by such nitrogen-containing supplements.

The independence of the endogenous and exogenous types of nitrogen metabolism is thus confirmed.

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EFFECT OF COOKING UPON THE THIAMIN CONTENT OF FOODS

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The extent to which foods lose thiamin during cooking is not very well known, as may be seen readily by a brief survey of the tables recently compiled by Fixsen ('38). Although thiamin has long been considered relatively unstable, evidence now indicates that cooking destroys less of this vitamin than was formerly supposed.

The present experiments were conducted to determine the effect of different cooking methods on the thiamin content of a representative variety of foods. Raw and cooked foods as well as the liquid drained from the cooked products were assayed.

The meat, cereal products, dried beans, potatoes (baked), and spinach were prepared for feeding once per week; the carrots, potatoes (boiled), snap beans, and green peas were cooked twice each week. With the exception of the dried beans, cereals and potatoes, the foods were cooked immediately after reaching the laboratory. All of the foods were stored in refrigerators in tightly covered containers until used. With the exception of the carrots and spinach, which were stored at freezing temperatures during their interval of use, all of the other foods, raw and cooked, were stored in a refrigerator kept at a temperature not to exceed 5°C. All of the cooked foods were ground, cut, or mashed for feeding before refrigeration; the raw vegetables, excepting carrots and spinach, were finely chopped just prior to feeding.

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[1] Transferred to National Bureau of Standards, U. S. Dept. Commerce, February 8, 1940.

[2] Transferred to Food and Drug Administration, U. S. Dept. Agriculture, November 15, 1939.

The thiamin content of the foods was determined by the rat-growth method of assay outlined by Booher and Hartzler ('39) employing crystalline thiamin hydrochloride as a standard. The basal diet designated as no. 111 consisted of casein (extracted with 60% alcohol) 18, agar 2, autoclaved yeast 15, Osborne and Mendel salts 4, cod liver oil 4, cottonseed oil 6, and cornstarch 51 parts. Before beginning an assay a few exploratory animals were given different amounts of the test food for the purpose of obtaining a rough indication of its thiamin potency.

The quantities of the test food for the assay proper were then selected with an aim to give growth rates just over and under that attained by 3 micrograms (1 International Unit) of thiamin hydrochloride. After the rats ceased growing, which required approximately 14 days, groups consisting of six to eight animals were given daily, in addition to the basal diet, the two predetermined levels of the test food and 3 micrograms of the thiamin hydrochloride, respectively.

Each animal in the group receiving thiamin had a littermate of the same sex and comparable weight in both groups receiving the test food. Animals of another group, littermates of those in the first three groups, were continued without supplement and thus served as negative controls. The quantity of supplement necessary to give exactly the same growth as 3 micrograms of the standard was finally determined by interpolation.

Preparation and sampling

Carrots. Long pointed carrots of a very deep orange color, grown in Texas and California, were purchased on the open market in Washington, D. C., at intervals from June until February. The carrots were scrubbed, dried on paper towels, scraped, and halved lengthwise. One set of halves was grated and fed raw; the other was cooked. In the pressure cooker method the halves were placed without added water in a small covered pan which was set on the rack of the cooker. After exhausting the cooker, the pressure was brought up to 15 pounds

(250°F.) as rapidly as possible (1 to 2 minutes required) and held at that pressure for a period of 11 minutes for small halves, 13 minutes for medium, and 15 minutes for large halves. In the boiling method the carrot halves were dropped into a small amount of boiling water, 100 ml. of water to 300 gm. of carrot, and boiled gently in a covered pan until tender, on the average 23 minutes. In both cases the cooked carrots were transferred to a glass bowl and chopped very fine with a two-bladed curved chopping knife.

Potatoes. Irish Cobbler potatoes, grown in Maine and selected for sizes weighing 150 to 200 gm., were boiled and baked. In the boiling method, whole pared potatoes were dropped into sufficient boiling salted water to cover them and gently boiled in an uncovered pan until tender, an average of 36 minutes being required. The cooked potatoes were mashed and thoroughly mixed. The cooking liquid was made up to such volume that 1 ml. represented 1 gm. of raw potato.

Unpared potatoes were baked at an oven temperature of 190°C. (375°F.) until soft, or an average of 63 minutes. After cooling they were removed from their skins, mashed, and thoroughly mixed. The skins were cut finely with scissors and proportionate amounts fed along with the mashed pulp.

Pared raw potatoes were grated and mixed, and weighed supplements were fed for comparison with the boiled potatoes. Unpared raw potatoes, after grating and mixing, were assayed for comparison with the thiamin content of the baked tubers.

Spinach. Bloomsdale Savoy spinach, purchased on the local market, was washed in four changes of water and then tossed on towels until visible moisture had been absorbed. Waste, consisting of roots, very coarse stems, withered or decayed leaves, and occasional bud stalks, was removed. A 500 gm. quantity of cleaned, dried, waste-free spinach was dipped in water and drained until just 100 gm. of water was left clinging to the leaves, which were then cooked with no additional water. The spinach was heated in a covered saucepan at moderate

heat for 2 minutes when the leaves began to wilt. Then the cover was removed and the spinach boiled gently for 7 minutes. The cooked spinach was drained and finely chopped before feeding. The water drained from the cooked leaves was made up to volume so that 1 ml. represented the amount from 5 gm. of uncooked spinach. Cleaned raw spinach chopped fine in a glass bowl with a food chopper was assayed for comparison with the cooked product.

Green peas. Laxton Progress peas, grown in the State of Washington, were purchased on the open market in Washington, D. C. After shelling, washing, and drying on paper towels, 500 gm. of peas were dropped into approximately 250 ml. of boiling water and simmered with occasional stirring in an uncovered vessel for 12 minutes.

Additional peas were cooked with soda; 0.3 gm. (1/16 teaspoon) of sodium bicarbonate per 500 gm. of peas was dissolved in the water before the addition of the vegetable. This method preserved only a slightly more intense green color than simmering in the absence of soda and did not affect the flavor of the peas.

Both cooked and raw peas were chopped finely before being fed. The cooking water from the peas was made up to volume so that 1 ml. represented the quantity in which 2 gm. of raw peas had been cooked. The cooking liquid from peas without soda had an average pH of 6.4; with soda the average pH was 7.4.

Snap beans. Bountiful variety snap beans, grown at the National Agricultural Research Center, Beltsville, Maryland, were used in this test. After discarding blossom and stem ends the beans were washed, dried on paper towels, broken in half, and added to sufficient boiling salted water to cover, approximately 450 ml. per 300 gm. of beans. They were boiled in a covered pan for 1 minute; the cover was then removed and boiling continued for a total of 40 minutes. The cooking liquid showed an average pH of 5.8.

Beans were also cooked in the same manner as described above except for the addition of 0.3 gm. of sodium bicarbonate

per 300 gm. of beans. This amount of soda, placed in the water before the addition of the beans, preserved green color but did not affect flavor. The cooking liquid had an average pH of 6.6.

In each case the liquid drained from the cooked beans was made up to volume for feeding so that 1 ml. represented the quantity in which 3 gm. of beans had been cooked.

Navy beans. Dried navy beans purchased on the open market were soaked for 16 hours in water (1 cup of beans to 2 cups of water). The water was drained off and the beans were dropped into boiling salted water (3 cups water to 1 cup dry beans) and were boiled gently until soft. This required an average time of 85 minutes.

Except for the addition to the cooking water of 0.4 gm. of sodium bicarbonate (a small "pinch") per cup of dry beans, the same method was used for cooking beans with soda. This amount of soda reduced the cooking time approximately one-third.

The cooked beans were mashed to a thick paste with the remaining small amount of cooking liquid for supplement feeding. The raw dry beans were ground in a food grinder until practically pulverized before feeding.

The liquid drained from the beans which had soaked overnight had an average pH of 6.5. Liquid from the beans cooked without soda had a pH of 5.7; liquid from those cooked with soda showed a pH of 6.0.

Rolled oats. Commercially packaged rolled oats, purchased in Washington, D. C., were used. One cup of the oats was added to 2 cups of boiling salted water and stirred and cooked for 2 minutes over an open flame. Cooking was continued in a double boiler for a total of 2 hours.

Whole wheat. A sample of hard red spring wheat grown near Great Falls, Montana, was cooked both as a cereal and as bread. For use as a cereal 1 cup of wheat, after being cracked, was added to $2\frac{1}{2}$ cups of boiling salted water, boiled and stirred for 2 minutes over an open flame, and finally cooked for an additional 28 minutes in a double boiler.

To test the effect of baking on the thiamin content of wheat, part of the whole wheat was ground into flour and a water bread containing no other flour was prepared. The bread was baked for a total period of 45 minutes; the baking temperature for the first 15 minutes was 218°C. (425°F.); for the remaining 30 minutes the temperature was reduced to 190°C. (375°F.). After baking, the loaf was sliced, dried at a moderate temperature (approximately 45°C.), and ground to fine crumbs for feeding.

Pork. Eight-rib sections of pork loin were purchased weekly on the open market, trimmed, and one thick chop with rib bone was cut from each end. The six-rib section remaining was prepared as a roast. The two chops were used to estimate the relative proportion of lean in the whole piece in order to relate raw and cooked weights. The lean from these two chops was ground twice, and used for the raw pork assay.

The loin roasts were cooked at an oven temperature of 175°C. (350°F.) until the internal temperature of the roasts reached 84°C. (183°F.), and the lean portion removed and ground twice for the assay.

The drippings from the roast, washed from the pan with small quantities of water, were chilled, freed from fat, made up to volume, and fed.

Subsequently another weekly series of four-rib sections of pork loin were purchased. After determining the relative proportion of lean in two of the chops as in the above experiment, the lean portion was ground and fed raw. The remaining two chops were braised, i.e., browned on each side in an uncovered skillet, and the cooking continued with the skillet covered, until the meat was well done. The lean meat from the braised chops was ground twice and fed.

RESULTS AND DISCUSSION

No loss of thiamin was observed in carrots prepared either by the boiling or pressure cooker methods. This is contrary to the findings of Richardson and Mayfield ('32) who reported a 20% loss of vitamin B_1 in cooking (boiling) carrots. These

same investigators found that stored potatoes also suffered a 20% loss of thiamin during boiling.

Baker and Wright ('35) found that potatoes boiled in their skins contained 0.3 International Units per gram as compared with 0.4 International Units per gram in samples of raw potatoes. The present studies indicated a 33% loss of thiamin in the boiled pared potato. Twenty per cent of this had been destroyed while about 13% had been dissolved in the cooking liquid. Baking caused 16% destruction of thiamin in potatoes.

During cooking the spinach lost 30% of its thiamin value; 22% of the total thiamin content was destroyed and 8% was recovered in the cooking liquid. Roscoe ('30) found that spinach boiled 15 minutes lost about half of its vitamin B_1 content in the cooking liquid. Hoff ('33) reported more than 50% loss of vitamin B_1 in spinach cooked by "ordinary household methods." Excessive vitamin B_1 losses reported by some investigators may have been the result of cooking in too large quantities of water or of employing too long a cooking time.

Green peas simmered without the use of soda retained 80% of their thiamin; peas simmered with a small amount of soda retained only 67% of this vitamin. In both cases 11% of the thiamin was dissolved in the cooking liquid. There was a 22% destruction of the vitamin in the presence of sodium bicarbonate, more than twice as much as was destroyed when no soda was used. Baker and Wright ('38) have reported thiamin values for fresh peas ranging from 1.6 to 2.8 International Units per gram; those of cooked fresh peas 0.8 to 1.2 International Units. Rose and Phipard ('37) found a 26% loss of vitamin B_1 in cooking peas for 15 minutes.

Snap beans cooked without soda retained about 68% of their original thiamin content. Eighteen per cent was destroyed and 14% dissolved in the cooking liquid. Approximately 60% of the thiamin in snap beans cooked with a "pinch" of soda was destroyed. About 40% was retained by the beans; only a slight trace was found in the cooking

liquid.   Mayfield and Richardson ('39) found no loss in thiamin value after cooking snap beans 45 minutes.   They found 0.30 International Units of thiamin per gram in raw beans, 0.33 International Units per gram in the cooked beans.

Navy beans cooked in tap water with or without soda retained all of their original thiamin value.   Soda shortened the time of cooking by a little more than one-third, or approximately 30 minutes.   The effect of cooking with soda for 30 minutes longer was not determined inasmuch as this would have no practical value.   Lantz ('38) found about 50% loss in the thiamin content of dried pinto beans soaked 16 hours and cooked 2¾ hours in distilled water.   This loss was greater when tap water was used or when a small amount of sodium bicarbonate was added.   Part of the loss reported by this investigator may be accounted for by the fact that the cooked beans were dried, ground, and stored before testing.   Mickelsen, Waisman and Elvehjem ('39) found that drying (animal tissues) under vacuum at temperatures above 70°C. caused appreciable destruction of vitamin B_1.

The preparation of whole grain wheat or oat cereals in the manner described caused no destruction of the thiamin content.   Wheat prepared as bread, however, lost about 15% of its thiamin.   This comparatively small loss is in agreement with the findings of Copping and Roscoe ('37), Morgan and Frederick ('35), and Scheunert and Schieblich ('37), all of whom report very little or substantially no destruction in the thiamin value of the original flours in the baking of bread.

Pork cooked as a roast lost 43% of its thiamin value.   Only a trace of thiamin was recovered in the drippings.   Braising caused about 15% destruction of this vitamin.   Christensen, Latzke and Hopper ('36) reported a 12% destruction of vitamin B_1 in pork which had been ground and subsequently stirred and cooked in a double boiler until it reached a constant temperature of 90°C.   This was dried and ground for assay.   Mickelsen, Waisman, and Elvehjem ('39) found a thiamin destruction of 50% caused by roasting pork loin and a 35% loss due to frying.   This is comparable to our findings

TABLE 1

Thiamin in raw and cooked foods and losses during cooking

FOOD	COOKING METHOD	TIME OF COOKING	AVERAGE COOKED WT. OF 100 GM. RAW FOOD	THIAMIN CONTENT					
				Per 100 gm. cooked food	Per 100 gm. raw food	In cooked food per 100 gm. raw wt.	Retained by cooked food	Dissolved in cooking liquid	Destroyed during cooking
		min.	gm.	I.U.	I.U.	I.U.	%	%	%
Carrots [1]	Pressure cooker	11–15	90	20	19	18	100	—	0
	Boiled	23±3 [2]	95	25	22	24	100	0	0
Potatoes	Baked	63±5 [2]	85	50	50	42	84	—	16
	Boiled (pared)	36±5 [2]	95	32	45	30	67	13	20
Spinach	Boiled	9	80	38	48	38	79	8	22
Peas, green	Simmered	12	97	124	150	120	80	11	9
	" with soda	12	99	100	150	100	67	11	22
Beans, snap	Boiled	40	85	18	22	15	68	14	18
	" with soda	40	85	11	22	9	41	Trace	59
Beans, navy	Boiled	83±22 [2]	320	75	250	240	100	—	0
	" with soda	53±4 [2]	330	80	250	265	100	—	0
Oats, rolled	Double boiler	120	635	33	200	210	100	—	0
Wheat; whole	Double boiler	30	485	36	180	175	100	—	0
	Baked (bread)	45	155	100	180	155	86	—	14
Pork loin (lean portion)	Braised (chop)	13±1 [2]	75	550	485	413	85	—	15
	Roasted	43±3 [2] per lb	65	400	455	260	57	Trace	43

[1] The assays on carrots were conducted under the direction of Dr. Hazel E. Munsell.

[2] Average deviation.

for roasting but their frying method appears to have caused an appreciably greater destruction of thiamin than the braising method used in these experiments.

The findings of the present experiments are summarized in table 1. Some interesting practical facts come to light in an analysis of these data. The whole-grain cereals and dried legumes included in every list of thiamin-rich foods furnish less of this vitamin to the diet than is generally supposed. In the past these lists have been made up on the basis of raw foods and the large proportion of water absorbed during preparation leaves an average serving of the cooked product with a relatively lower thiamin value than is ordinarily realized. On a serving basis baked potatoes, including skin, rank higher in thiamin than cooked whole wheat or oat cereals. Even boiled potatoes, spinach, and carrots may be classed as furnishing amounts of thiamin per serving comparable to those supplied by these cooked cereals. One slice of an all-whole-wheat bread also contains approximately the same quantity of thiamin as one serving of these vegetable and cereal foods. A rough estimate of ten servings of these foods would be needed to meet the liberal daily allowances often recommended (Cowgill, '38). Navy beans, taking up less water during cooking, furnish proportionately more thiamin per serving than the cereals.

The seeds in snap beans as they are usually eaten are too immature to make this vegetable a rich source of thiamin. Green peas, on the other hand, supply in a single serving about one-fourth to one-third of the quantity of thiamin usually recommended for a liberal daily allowance.

In contrast to the other foods which were analyzed, one serving of the lean portion of pork loin cooked either as chop or roast furnishes this plentiful daily allowance of thiamin for the adult.

SUMMARY

Thiamin losses due to different cooking procedures were determined by the rat-growth method for a representative variety of foods. Experiments were set up to show the per-

centage of the thiamin originally present in the raw food that was (1) retained by the cooked product, (2) dissolved in the cooking water, and (3) destroyed.

Thiamin destruction amounted to as much as 22% in some vegetables boiled in water and additional amounts up to 15% dissolved in the cooking water. In cases where the cooking water is discarded total thiamin losses in vegetables may amount to approximately 20 to 35%. The addition of a small amount of sodium bicarbonate markedly increased the destruction of thiamin in green peas and snap beans but had no significant effect upon the thiamin content of boiled navy beans. Roasting caused a loss of 43% of the thiamin in pork loin, nearly three times as much destruction as braising. Double boiler cooking of whole grain cereals did not destroy thiamin; baking bread caused about 15% loss of this vitamin. The relative values of the different cooked foods in meeting the daily human requirements for thiamin are discussed.

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THE METABOLISM OF TYROSINE, ASPARTIC ACID AND ASPARAGINE, WITH SPECIAL REFERENCE TO RESPIRATORY EXCHANGE AND HEAT PRODUCTION [1]

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In a previous paper (Kriss, '39) determinations of the metabolizability of glutamic acid, glycine and alanine were reported, based on balances of nitrogen, carbon and energy, and the relationships between the urinary nitrogen, respiratory exchange and metabolizable energy for each of these amino acids—factors usable in indirect calorimetry for the computation of heat production—were determined. These factors were found to be closely correlated with the ratios of carbon to nitrogen in the different amino acids. It seemed desirable to extend the study to other amino acids; and the present paper presents the results obtained with the amino acids tyrosine, aspartic acid and asparagine.

EXPERIMENTAL

The experimental procedure employed in this investigation was, in most respects, the same as that followed in the previous study (Kriss, '39). Sixteen male albino rats weighing approximately 200 gm. each served as the experimental subjects. The animals were first placed on a basal ration of 8 gm. per day, this ration consisting of 93.7% of an approximately complete calf meal (Forbes, Kriss and Miller, '34) and 6.3% of butterfat. This diet was adequate for maintaining

the animals in approximate equilibrium of nitrogen and energy. The animals were kept on the ration for periods of from 2 to 3 weeks, and feces and urine were collected from each rat during the final 7-day period.

Following this treatment six of the animals received in addition to the basal ration 1.288 gm. of l-tyrosine per day; five received 2 gm. of l-aspartic acid, and five received 2 gm. of l-asparagine as the daily supplements. These supplemented diets were fed to the rats for a period of 8 days. Feces and urine were collected during the last 5 days.

During the entire experimental period the food was offered the animals in equal portions twice a day, as in the previous studies.

TABLE 1

Analysis of basal ration and of amino acid supplements

MATERIAL	MOISTURE	NITROGEN	CARBON	ENERGY
	%	%	%	*Calories per gram*
Basal ration	10.38	3.25	42.26	4.306
l-tyrosine	0.29	7.62	58.74	5.824
l-aspartic acid	0.05	10.34	36.19	2.888
l-asparagine	12.03	18.28	31.97	3.011

The basal ration and the amino acid supplements [2] were analyzed for nitrogen, carbon, energy and moisture. The urine and feces were analyzed for energy, carbon and nitrogen. Energy was determined in the urine composited for each group of animals, while nitrogen and carbon were determined in the urine of each individual rat. The feces were composited for each group before being analyzed. As in the previous experiments, the excretory products representing the amino acid supplements were determined as the differences between the residues from the basal ration and the supplemented diets.

RESULTS

The analyses of the basal ration and of the amino acids used as supplements are presented in table 1. The large

[2] Purchased from Eastman Kodak Co., Rochester, N. Y.

percentage of moisture in the asparagine corresponds almost exactly to 1 molecule of water of crystallization. The percentages of nitrogen and carbon in all three amino acids, expressed on the dry matter basis, differ by less than 2% from the theoretical values.

The results of the urinary analysis, presented in table 2, show in a consistent and significant manner, as in the previous study, the influence of the amino acid feeding on the nitrogen and carbon content of the urine and, especially, on the $C:N$ and energy:N ratios. The average ratio of carbon to nitrogen in the urine from the basal ration is 0.77:1. Supplementing the basal diet by 1.288 gm. of tyrosine raised the ratio of carbon to nitrogen in the urine to 1.61:1. On the other hand, the addition to the basal diet of 2 gm. of either aspartic acid or asparagine had the effect of slightly lowering the $C:N$ ratios of the urine.

The urinary $C:N$ ratios representing aspartic acid (0.50:1) and asparagine (0.45:1) are only slightly higher than the $C:N$ ratios of urea (0.43:1). Similarly, the urinary energy:nitrogen ratios for aspartic acid (6.30:1) and for asparagine (5.80:1) are only slightly higher than the ratio of energy to nitrogen found in urea (5.43:1) (Kriss and Marcy, '40). However, tyrosine is represented by ratios of carbon to nitrogen (2.77:1) and energy to nitrogen (25.18:1), in the urine, which are several times as high as the corresponding ratios existing in urea. These $C:N$ and calories:N ratios indicate that nearly all of the urinary nitrogen derived from aspartic acid or asparagine represents metabolized amino acid, and that a relatively large proportion of tyrosine is eliminated in the urine in unmetabolized or incompletely metabolized form. A significant fact in this connection is that the urine from the tyrosine-supplemented ration was nearly black, indicating the presence of homogentisic acid.

We have not found it convenient during the experiments to determine directly the urea nitrogen and the amino acid nitrogen in the urine. We have, therefore, followed the method outlined in the previous publication (Kriss, '39) of estimat-

TABLE 2

Urinary analysis

RAT NO.	BASAL RATION				BASAL RATION PLUS 1.288 GM. TYROSINE				DIFFERENCES DUE TO AMINO ACID				
	Nitrogen per day	Carbon per day	C:N ratio	Energy per day	Nitrogen per day	Carbon per day	C:N ratio	Energy per day	Nitrogen	Carbon	C:N ratio	Energy	Energy:N ratio
	mg.	*mg.*		*cal.*	*mg.*	*mg.*		*cal.*	*mg.*	*mg.*		*cal.*	
41	134	114	0.85		264	428	1.62		130	314	2.42		
42	153	117	0.77		276	428	1.55		123	311	2.53		
43	146	111	0.76		266	442	1.66		120	331	2.76		
44	168	128	0.76		264	438	1.66		96	310	3.23		
45	165	127	0.77		274	418	1.53		109	291	2.67		
46	153	110	0.72		256	418	1.63		103	308	2.99		
Average	153	118	0.77	1256	267	429	1.61	4126	114	311	2.77	2870	25.18
					BASAL RATION PLUS 2 GM. ASPARTIC ACID								
51	156	134	0.86		327	218	0.67		171	84	0.49		
52	187	145	0.78		316	218	0.69		129	73	0.57		
53	194	141	0.74		296	193	0.65		102	52	0.51		
54	168	122	0.73		337	192	0.57		169	70	0.41		
55	143	114	0.80		298	199	0.67		155	85	0.55		
Average	170	131	0.77	1387	315	204	0.65	2301	145	73	0.50	914	6.30
					BASAL RATION PLUS 2 GM. ASPARAGINE								
56	138	110	0.80		434	243	0.56		296	130	0.44		
57	157	120	0.76		460	265	0.58		303	145	0.48		
58	158	122	0.77		483	265	0.55		325	143	0.44		
59	175	129	0.74		490	267	0.55		315	138	0.44		
60	178	129	0.73		460	255	0.55		282	126	0.45		
Average	161	122	0.76	1279	465	259	0.56	3041	304	136	0.45	1762	5.80

ing the portions of the urinary nitrogen which represent metabolized and unmetabolized amino acid, respectively (table 3), on the basis of the C: N and energy: N ratios. There is evidence (table 5), which will be discussed later, to show that under the existing experimental conditions, this method of partitioning the urinary nitrogen yields correct values for the quantities of amino acid metabolized.

TABLE 3

Metabolizability of amino acids

	ASPARTIC ACID 2.0 GM.		ASPARAGINE 2.0 GM.		TYROSINE 1.288 GM.
Income					
Energy, calories	5776		6022		7501
Carbon, mg.	724		639		757
Nitrogen, mg.	207		366		98
Outgo					
Feces					
Energy, calories	389		318		349
Carbon, mg.	35		28		21
Nitrogen, mg.	10		12		3
Urine	AS DETERMINED	CORRECTED TO N-EQUILIBRIUM	AS DETERMINED	CORRECTED TO N-EQUILIBRIUM	CORRECTED TO N-EQUILIBRIUM
Energy, calories	914	1242	1762	2052	2714
Carbon, mg.	73	99	136	158	296
Total nitrogen, mg.	145	197	304	354	95
Unmetabolized { mg.[1]	4	5	5	6	35
amino acid N { mg.[2]	6	8	10	12	31
{ mg.[1]	141	192	299	348	60
Metabolized { mg.[2]	139	189	294	342	63
amino acid N { %[1]	97	97	98	98	63
{ %[2]	96	96	97	97	66
Metabolizable					
Energy, calories	4473	4145	3942	3652	4438
Carbon, mg.	616	590	475	453	440
Energy, %	77.4	71.8	65.5	60.6	59.2
Carbon, %	85.1	81.5	74.3	70.9	58.1

[1] Computed on the basis of C: N ratio.

[2] Computed on the basis of energy: N ratio.

The balances of nitrogen, carbon and energy for the different amino acids are presented in table 3. The data for feces and urine are the average values for the respective groups of animals. The data for total urinary nitrogen, carbon and energy representing aspartic acid and asparagine, and consequently, the values for metabolizable energy and carbon of these amino acids are given on two bases: (1) as determined and (2) corrected to represent nitrogen equilibrium. The corrections of the energy and carbon of the urine to nitrogen equilibrium were based on the relationships, as directly determined, between the urinary energy and nitrogen and between the urinary carbon and nitrogen, respectively. In other words, the determined values for urinary energy and carbon were increased in the same proportion as the nitrogen of the urine had to be increased to represent nitrogen equilibrium. The directly determined balances of nitrogen indicate that of the total nitrogen ingested as aspartic acid and as asparagine 25.1% and 13.7%, respectively, were retained in the body.

In the case of tyrosine feeding, however, the increase in elimination of urinary nitrogen (114 mg.) caused by this supplement (see table 2) exceeded somewhat the nitrogen content of the latter (98 mg.), thus indicating that none of the tyrosine was retained in the body. In order to compute the relationship between the tyrosine ingested and the products eliminated, the data for urinary nitrogen, carbon and energy for this supplement were corrected, as in table 3, to a basis of nitrogen equilibrium, this correction being made on the assumption that the excess of nitrogen eliminated in the urine during the period of feeding the tyrosine-supplemented diet was derived from the protein of the basal ration rather than from body protein, inasmuch as the total nitrogen balance during this period was slightly positive ($+15$ mg.).

In giving the foregoing interpretations the writers are not unaware of the recent study of tyrosine metabolism by Schoenheimer, Ratner and Rittenberg ('39), who used isotopic nitro-

gen, and found that a considerable part of the labeled nitrogen of dietary tyrosine was transferred to and deposited in the tissue proteins. The finding that samples of pure tyrosine isolated from liver proteins and from body proteins contained a high concentration of labeled nitrogen was rightly considered by the authors as indicating that some dietary tyrosine was directly deposited in the tissue proteins. They have also observed that while the nitrogen of the dietary tyrosine was only partly excreted in the urine, an equivalent amount of protein nitrogen was excreted, thus effecting nitrogen equilibrium.

These observations of Schoenheimer and co-workers are interesting from the point of view of intermediary metabolism, and serve to emphasize the fact that balances of nitrogen do not reveal the intimate character of the intermediary reactions.

Insofar as the results of the present study are concerned, it should be borne in mind that they deal chiefly with the ultimate effects as shown by the balances of nitrogen, carbon and energy, and that the interpretations of these results are presented largely from this point of view.

The results of partitioning the urinary nitrogen (table 3) into fractions representing metabolized and unmetabolized amino acid show, in accord with the $C:N$ ratios and energy: N ratios of the urine, that tyrosine was much less completely metabolized than was either aspartic acid or asparagine. Calculated on the basis of $C:N$ ratios, the percentages of the total nitrogen excreted in the urine representing metabolized amino acid are 63 for tyrosine, 97 for aspartic acid, and 98 for asparagine. Values close to these were obtained when the calculation was based on the energy: N ratios.

Of the total energy ingested as tyrosine 59.2% was metabolizable. The metabolizable energy values of aspartic acid and of asparagine (corrected to a basis of nitrogen equilibrium) were found to be 71.8% and 60.6%, respectively, of the gross energy of the amino acids.

The carbon was found to be metabolizable (on a N-equilibrium basis) to the extent of 58.1% for tyrosine, 81.5% for aspartic acid, and 70.9% for asparagine.

The relatively low metabolizability of the carbon and energy of asparagine, as compared with aspartic acid, was associated with the greater nitrogen content of the former. The low metabolizability of the carbon and energy of tyrosine was apparently due to the excretion in the urine of a relatively large proportion of this amino acid in unmetabolized form.

Table 4 sets forth the computations of the respiration and energy factors for the different amino acids. Attention is called to the facts that in this table, as in the corresponding table of the previous publication (Kriss, '39), the data representing the intake are on the basis of pure amino acids, and that the data for urinary constituents and, consequently, for metabolizable products are on the basis of nitrogen equilibrium.

The values for carbon and energy of the urine were computed in relation to the urinary nitrogen, on the basis of the data given in table 3. The values for fecal nitrogen, carbon and energy were computed in proportion to the nitrogen intake from the data of table 3. The values for hydrogen and oxygen of the feces were computed from the fecal nitrogen on the assumption that the latter represents the unabsorbed portion of the amino acid. The values for hydrogen and oxygen of the urine were computed from the calculated values of urea and unmetabolized amino acid present. The important relationships derived in table 4 are given in the lower part of the table.

The values for O_2, CO_2 and calories per gram of urinary nitrogen are approximately twice as great for aspartic acid as the corresponding values for asparagine. The same factors for tyrosine are approximately four times as great as those for asparagine. As in the previous study with other amino acids, these factors show a close correlation with the ratios of carbon to nitrogen in the materials, these ratios being,

TABLE 4

Determination of factors for computing the respiratory exchange and the heat production in the metabolism of tyrosine, aspartic acid and asparagine

	TYROSINE	ASPARTIC ACID	ASPARAGINE
Intake, computed per 100 gm. of pure amino acids			
Energy, Calories	584.1	288.9	342.3
Nitrogen, gm.	7.73	10.52	21.20
Carbon, gm.	59.67	36.07	36.34
Hydrogen, gm.	6.08	5.26	6.06
Oxygen, gm.	26.52	48.10	36.34
Outgo			
Feces			
Energy, Calories	27.9	19.8	18.4
Nitrogen, gm.	0.24	0.51	0.70
Carbon, gm.	1.66	1.78	1.62
Hydrogen, gm.	0.19	0.26	0.20
Oxygen, gm.	0.82	2.33	1.20
Urine			
Energy, Calories	214.0	63.1	118.8
Total nitrogen, gm.	7.49	10.01	20.50
Unmetabolized amino acid N	2.60	0.35	0.51
Metabolized (urea) N, gm.	4.89	9.66	19.99
Carbon, gm.	23.34	5.04	9.17
Hydrogen, gm.	2.75	1.56	3.01
Oxygen, gm.	11.71	7.12	12.39
Metabolized			
Energy, Calories	342.2	206.0	205.1
Carbon, gm.	34.67	29.25	25.55
Hydrogen, gm.	3.14	3.44	2.85
Oxygen, gm.	13.99	38.65	22.75
Intramolecular H_2O			
Hydrogen, gm.	1.75	3.44	2.84
Oxygen, gm.	13.99	27.52	22.75
Carbon oxidized to CO_2, gm.	34.67	29.25	25.55
Hydrogen oxidized to H_2O, gm.	1.39	0.00	0.01
CO_2 produced, gm.	127.12	107.25	93.68
O_2 required to oxidize C, gm.	92.45	78.00	68.13
O_2 required to oxidize H, gm.	11.12	0.00	0.08
Intramolecular O_2 in excess of that required to oxidize H, gm.		11.13	
Total O_2 required, gm.	103.57	66.87	68.21
Respiratory quotient	0.89	1.17	1.00
Liters O_2 required per gm. urinary N	9.68	4.68	2.33
Liters CO_2 produced per gm. urinary N	8.64	5.46	2.33
Calories metabolized per gm. urinary N	45.7	20.6	10.0
Calories per liter O_2	4.72	4.40	4.30
Liters O_2 required per gm. N of amino acid metabolized	14.82	4.84	2.39
Liters CO_2 produced per gm. N of amino acid metabolized	13.24	5.66	2.39
Calories metabolized per gm. N of amino acid metabolized	70.0	21.3	10.3

for tyrosine 7.72 : 1, for aspartic acid 3.43, and, for asparagine 1.71.

The respiration and energy factors for tyrosine, expressed per gram of nitrogen of amino acid metabolized, are considerably higher than the corresponding values expressed per gram of total urinary nitrogen. With the other two amino acids the respective differences between the two sets of values are very slight. This is a result of the calculation (table 3) that only very small quantities of unmetabolized aspartic acid or asparagine appeared in the urine, and that a relatively large quantity of tyrosine was excreted in the urine in the unmetabolized form.

It is obvious from the foregoing results that the total urinary nitrogen is not always a true index of the metabolism of amino acids, and that the respiration and energy factors expressed per gram of nitrogen of amino acid metabolized can be applied with safety only when the quantity of completely metabolized amino acid is definitely known. The determined factors expressed per gram of total urinary nitrogen apply correctly to the experimental conditions. The applicability of these factors to other conditions of experimentation would depend on the constancy of the unmetabolized fraction of any particular amino acid appearing in the urine. It is noteworthy that in previous experiments with rats the levels of intake of heart muscle protein, casein and gelatin (Kriss and Voris, '37) and of glycine (Kriss, '39) had but a slight effect on the respiration and energy values per gram of urinary nitrogen; and that the proportions of glycine, alanine and glutamic acid, respectively, found by us to be excreted in unmetabolized form in the urine of rats are in substantial agreement with those found by Lusk ('12–'13) in the dog.

Table 5 presents a comparison between the determined and the theoretical values for the gaseous exchange and heat production per gram of nitrogen of amino acid metabolized. This table includes results obtained with other amino acids in the previous study.

The determined values are those derived in table 4, which involved the use of the computed fractions of urinary nitrogen representing amino acid metabolized, the latter fractions being the average values as calculated on the basis of C: N and energy: N ratios. The theoretical values represent the gaseous exchange and the heat of complete combustion of the amino acids minus the corresponding values of urea that can be calculated to form from them, in relation to their nitrogen content.

TABLE 5

The determined as compared with theoretical values for the gaseous exchange and heat production per gram of nitrogen of amino acid metabolized

AMINO ACIDS	HEAT PRODUCED PER GRAM N OF AMINO ACID METABOLIZED		CO$_2$ PRODUCED PER GRAM N OF AMINO ACID METABOLIZED		O$_2$ REQUIRED PER GRAM N OF AMINO ACID METABOLIZED	
	Determined	Theoretical	Determined	Theoretical	Determined	Theoretical
	Calories	*Calories*	*Liters*	*Liters*	*Liters*	*Liters*
Glutamic acid [1]	32.1	32.9	7.05	7.20	7.08	7.22
Glycine [1]	11.2	11.2	2.40	2.40	2.42	2.38
Alanine [1]	22.7	22.3	3.95	4.00	4.78	4.81
Tyrosine	70.0	70.1	13.24	13.62	14.82	15.21
Aspartic acid	21.3	22.0	5.66	5.60	4.84	4.80
Asparagine	10.3	10.7	2.39	2.40	2.39	2.40

[1] Reported previously (Kriss, '39).

The agreement between the determined and the theoretical values of table 5 is, indeed, very close for all items and for all amino acids, and cannot be considered as fortuitous. In the majority of the comparisons the differences are less than 2% of the theoretical values, and in only two cases do they exceed 3%. Of especial significance is the very close agreement between the values compared for alanine and for tyrosine, inasmuch as considerable quantities of these substances were calculated to be excreted in the urine in the unmetabolized form (table 3). Any large error in the use of the ratios of carbon to nitrogen and energy to nitrogen, especially in the case of alanine and tyrosine, would have shown up in these comparisons.

Insofar as the degree of agreement between the determined and the theoretical values depends on the fractions of urinary nitrogen calculated to represent metabolized amino acid, the very close agreement obtained indicates that the calculation of these fractions possesses a high degree of accuracy. It also indicates that the quantitative relationships between the nitrogen of the urine and its carbon and energy content, respectively, as determined for the amino acids by direct comparison of the urinary constituents from the basal ration with those from the amino acid-supplemented rations (table 2), correctly represent the amino acid supplements, as far as the ultimate metabolic effects are concerned.

SUMMARY

Tyrosine, aspartic acid and asparagine fed to rats as supplements to a mixed maintenance ration, in quantities supplying 7.5, 5.8 and 6.0 kilogram-calories per day, respectively, appeared to be absorbed to the extent of 97%, 95% and 97%. The balances of nitrogen indicated that of the total nitrogen ingested as aspartic acid and as asparagine 25.1% and 13.7%, respectively, were retained in the body, and that none of the tyrosine was retained. These balances were presumably conditioned by the composition of the basal ration.

The C:N ratios of the urinary constituents from the different supplements were as follows: from tyrosine 2.77:1; from aspartic acid 0.50:1; and from asparagine 0.45:1. The energy:N ratios of the urine derived from the supplements were as follows: from tyrosine 25.18:1; from aspartic acid 6.30:1; and from asparagine 5.80:1. On the basis of these ratios the calculated fractions of the urinary nitrogen representing metabolized amino acids were approximately, for tyrosine 65%, for aspartic acid 97% and for asparagine 98%. The metabolizable energy values of tyrosine, aspartic acid and asparagine (corrected to a basis of nitrogen equilibrium) were found to be 59.2%, 71.8% and 60.6%, respectively, of the gross energy of the amino acids.

Factors were determined for computing the respiratory exchange and the heat production in the metabolism of each of the amino acids tested. The values obtained for O_2, CO_2 and calories per gram of urinary nitrogen are approximately twice as great for aspartic acid as the corresponding values for asparagine; and the values for tyrosine are approximately four times as great as those for asparagine, thus showing, in confirmation of a previous report, a close correlation between these factors and the ratios of carbon to nitrogen in the materials.

A very close agreement was obtained between the determined respiration and energy factors, expressed per gram of nitrogen of amino acid metabolized, and the values computed on a theoretical basis.

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IRON UTILIZATION IN DOGS ON MILK DIETS [1]

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ONE FIGURE

Whole milk, unlike other diets which have been used in iron metabolism studies with dogs (Fontes and Thivolle, '36; Huebner and Frerichs, '37; Hahn et al., '39), is relatively low in iron. When proper precautions against iron contamination are taken, the amount of iron supplied in milk as the sole article of diet, is insignificant. In the course of our investigation it was determined that growing dogs consume about 140 cc. of whole milk per kilogram of body weight per day, and mature dogs slightly less. Iron analyses made on samples of milk taken at various times indicated that the iron intake due to the milk feeding was only about 1 mg. per dog per week.

When Potter, Elvehjem and Hart ('38) began their study of anemia in dogs on milk diets, they used 30 mg. of iron daily, a figure based on the earlier work of Schultze, Elvehjem and Hart ('36 a, '36 b) with pigs. Since calculation of the data of Potter et al. ('38) showed that less than one-fourth of the iron fed at the 30 mg. level was used to build blood hemoglobin (Hb), we concluded that the level was probably higher than was actually needed and that some of it was either stored or excreted. In order to study iron utilization more exactly it was important to know an approximate mini-

mal figure for the amount of iron which would allow both optimal growth and blood formation. In order to arrive at such a figure one must consider not only the amount of iron in blood hemoglobin in the dog at any definite time, but also the amount of iron which would be present in the dog's body as muscle hemoglobin iron and parenchyma iron. The following considerations were of value in this regard.

According to Hahn ('37), iron is distributed in the body of the normal dog in the following approximate percentages of the total: blood Hb iron 60%; muscle Hb iron 6%; parenchyma iron 15%; and storage iron 20%. The experimental data of Whipple ('26) and Hahn and Whipple ('36) indicate that the variation from these values is low in the cases of blood Hb iron and parenchyma iron, but may be high for muscle Hb iron and storage iron. If 30 mg. of dietary iron are completely absorbed and distributed as above in a dog's body, there will result about 5.3 gm. of blood Hb (0.1 mg. Fe $\backsim$ 0.294 gm. Hb). The daily blood Hb production of a puppy growing at the rate of 100 gm. per day and maintaining a blood Hb level of 15 gm. per 100 cc. of blood, however, is only about 1.2 gm. The dose of 30 mg. of iron is therefore theoretically many times higher than that needed by growing dogs of average sizes (adult weight 12 to 16 kg.). Even when the added requirement for iron in puppies severely anemic at weaning is calculated, this level is found to be many times too high.

In all calculations the blood was considered to be 8% of the body weight. Although the validity of this assumption is always open to question, consideration of the data will show that any probable error, due to unaccounted-for changes in blood volume, would hardly be so great as to change its significance.

In our studies the primary objective was to determine the effect of copper deficiency on the extent of iron utilization. We ('39), had already produced a copper deficiency of such severity that no response was shown to iron alone. Incidentally, during the course of the experiments evidence was obtained substantiating our earlier finding that cobalt in very small amounts may stimulate hematopoiesis.

EXPERIMENTAL

Dogs placed on raised screens in wooden cages at 4 weeks of age and fed whole milk only became severely anemic (Hb 3.5 to 6.0 gm. per 100 cc. of blood) in 3 to 9 weeks. At this point either 10 mg. of iron as $FeCl_3$ alone, or 10 mg. of iron plus 2 mg. of copper as $CuSO_4$ was fed. Manganese as a solution of $MnCl_2$ was fed at a level of 1 mg. per day to all dogs.

Two litters of four dogs each were made anemic as above and used in experiments to establish the following points: (1) How soon would a copper deficiency affect the rate of hemoglobin formation in dogs fed iron alone? (2) What level of iron would just meet the needs of the growing anemic dogs?
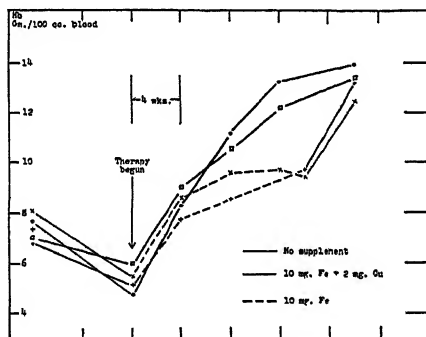
Fig. 1   Hemoglobin curves of four puppies allowed to become anemic on milk alone and then fed iron or iron plus copper.

Dogs fed iron alone for a period and finally a supplement of copper. Responses in four dogs fed 10 mg. of iron alone per day were typified by increases in blood Hb to a level of 8 to 10 gm. per 100 cc. of blood during the first week or two of therapy, after which no further increase occurred until copper was introduced (fig. 1). Calculations indicated that 80 to 90% of the iron ingested during the first week of therapy was converted to blood Hb. In succeeding weeks, however, almost none of the iron fed was returned as blood Hb. When copper was finally given to dogs which had ceased building Hb, there occurred an early resumption of the curve upward to normal levels. In all of these cases the amount of iron

ingested was considerably in excess of the amount theoretically needed. Thus it appeared that a copper deficiency became manifest within a week or two after the beginning of iron therapy. Figure 1 shows the typical Hb curves of some of the dogs fed iron alone and iron plus copper.

Dogs fed iron plus copper from the beginning of the test. Theoretically 10 mg. of iron per day will answer the needs of growing anemic dogs and allow some storage. Also, in the presence of adequate copper, this amount of iron would be expected to allow a maximal rate of hemoglobin regeneration. Each of four dogs receiving 10 mg. of iron and 2 mg. of copper daily showed rapid increases in blood Hb level during the first weeks of therapy. After the Hb level of the blood had reached 10 gm. per 100 cc., increases were much less rapid. In all cases the Hb level and the weight increases were greater than those shown by the four dogs receiving iron alone. Although calculated iron utilization approached 100% during the first few weeks of therapy, the per cent of utilization decreased rapidly thereafter. Thus iron utilization did not parallel iron ingestion and after 6 to 8 weeks of therapy only 60 to 70% of the total iron ingested could be accounted for. The most likely explanation seemed to be that the level of iron fed was in excess of the capacity of the dogs to utilize it and that the excess iron must have been either stored or excreted. The second possibility, that the small amounts of iron involved would not be excreted but would be efficiently stored appeared likely, and the following experiment was designed from this viewpoint.

Experiments with measured iron intake. The iron intake was limited to only that amount which would be expected to meet the needs of the animal for growth and Hb building and all of this iron was supplied during the first few weeks of therapy. The data obtained on two closely matched litter mate puppies are reported with all essential details in tables 1 and 2.

The per cent of iron appearing as blood hemoglobin iron in dogs 19 and 20 is in fair agreement with the figure of 60% found analytically by Hahn and Whipple ('36). The somewhat

higher values for our dogs may be accounted for by the lack of any storage iron.

Although dog no. 20, which did not receive copper during the early growth stage, showed the same per cent conversion of its iron to blood hemoglobin iron as did dog no. 19 which received copper, it should be borne in mind that dog no. 20 received only one-half as much iron as did dog no. 19 and maintained a consistently lower hemoglobin level in the blood.

<div align="center">

TABLE 1

Analysis of iron utilization in dog receiving a limited amount of iron together with copper during the early growth stage

</div>

DOG NO. 19   IRON UTILIZED DURING PERIOD %

A. Five weeks following weaning, dog was fed the milk diet + 10 mg. Fe and 2 mg. Cu daily. Essential data obtained are indicated below:

1. Weight at start of iron therapy = 3070 gm.
   Level of Hb in the blood = 3.6 gm. per 100 cc.

$$\frac{\text{Hb in dog}}{\text{at start}} = \frac{0.08 \times 3070 \times 3.6}{100} = 9 \text{ gm.}$$

2. Iron fed during 13-week period = 910 mg. equivalent to 268 gm. Hb (1 mg. Fe ⌣ 0.294 gm. Hb)

$$\text{Total body Hb at end of period} = \frac{0.08 \times 12400 \times 14.47}{100} = 143 \text{ gm.}$$

Hb made during period = 143 — 9 = 134 gm.

$$\text{Conversion of 910 mg. Fe to blood Hb} = \frac{100 \times 134 \text{ gm. Hb (actual)}}{268 \text{ gm. Hb (theoretical)}} = 50$$

B. Had any of the above iron been stored? Dog was again fed milk diet alone, and bled:

1. 920 cc. of blood ⌣ 111 gm. Hb removed in following 8-week period.

$$\text{Total body Hb at end of period} = \frac{0.08 \times 13300 \times 7.28}{100} = 77 \text{ gm.}$$

Hb made during period = 77 — (143 — 111) = 45 gm.

Total Hb made in 21 weeks = 134 + 45 = 179 gm. Hb

$$\text{Conversion of 910 mg. Fe to blood Hb} = \frac{100 \times 179 \text{ gm. (actual)}}{268 \text{ gm. (theoretical)}} = 67$$

2. Further bleeding resulted in loss of Hb, indicating depletion of iron stores.

3. Estimated total parenchyma Fe = ... 15

   Estimated total muscle Hb Fe = ... 6

   Estimated storage Fe = ... 0

4. Total iron accounted for = ... 88

Dog no. 19 received 2 mg. of Cu per day during the 13 weeks of therapy and 1 mg. of Mn per day throughout the experiment.

TABLE 2

Analysis of iron utilization in dog receiving a limited amount of iron but no copper during the early growth stage

DOG NO. 20. LITTERMATE TO DOG NO. 19

IRON UTILIZED
DURING PERIOD
%

A. Fe fed alone with milk to deplete Cu stores:
  1. Weight at start of Fe therapy = 2250 gm.
    Level of Hb in the blood = 4.6 gm. per 100 cc.

$$\text{Hb in dog at start} = \frac{0.08 \times 2250 \times 4.6}{100} = 8 \text{ gm.}$$

  2. Fe fed during first 3 weeks of 23-week period = 460 mg. ⌒ 135 gm. Hb.

$$\text{Total body Hb at end of period} = \frac{0.08 \times 10900 \times 10.97}{100} = 95 \text{ gm.}$$

    Hb made during period = 95 — 8 = 87 gm.

$$\text{Conversion of 460 mg. Fe to blood Hb} = \frac{100 \times 87 \text{ gm. Hb (actual)}}{135 \text{ gm. Hb (theoretical)}} = 64$$

B. Bleeding to induce use of any Fe stored during previous period:
    Dog maintained on milk alone during period
  1. 370 cc. of blood ⌒ 37 gm. Hb removed 3-week period

$$\text{Total body Hb at end of period} = \frac{0.08 \times 9600 \times 8.6}{100} = 66 \text{ gm.}$$

    Hb made during period = 66 — (95 — 37) = 8 gm.
    Total Hb made during 26 weeks = 86 + 8 = 94 gm.

$$\text{Conversion of 460 mg. Fe to blood Hb} = \frac{100 \times 94}{135} = \dots\dots\dots \quad 70$$

    The appearance of the dog at this stage was one of acute nutritional deficiency and the Cu level of the blood was only 30 to 40 mcg. per 100 cc.
  2. Further bleeding resulted in a proportionate loss of Hb.

C. Period of iron therapy:
  1. Fe fed during next 5-week period = 700 mg. ⌒ 206 gm. Hb.
    Conversion of Fe fed during period C

$$\text{to blood Hb} = \frac{100 \times 6 \text{ gm. Hb (actual)}}{206 \text{ gm. Hb (theoretical)}} \dots\dots\dots\dots\dots \quad 3$$

    Blood Hb remained at 8.5 gm. per 100 cc. and the dog remained apathetic.

D. Period of Fe + Cu therapy:
  1. Cu introduced at a level of 2 mg. per day for 3 weeks.
    At end of 8-week period 1120 mg. Fe fed ⌒ 329 gm. Hb.
    Conversion of Fe fed during periods C

$$\text{and D to blood Hb} = \frac{100 \times 47}{329} = \dots\dots\dots\dots\dots \quad 14$$

$$\text{Conversion of Fe fed during period D to blood Hb} = \frac{100 \times 41}{124} = \dots \quad 33$$

    Blood Hb level = 11 gm. per 100 cc.
    General condition markedly improved. Hair coat became darker.

E. Period of Fe + Cu + Co therapy:
  1. Co introduced at level of 0.5 mg. per day for 3 weeks
    At end of 11-week period 1540 mg. Fe fed ⌒ 453 gm. Hb.
    Conversion of Fe fed during periods C, D, and

$$\text{E to blood Hb} = \frac{100 \times 99}{453} = \dots\dots\dots\dots\dots\dots \quad 22$$

$$\text{Conversion of Fe fed during period E to blood Hb} = \frac{100 \times 52}{124} = \dots 42$$

Also dog no. 20 grew at a much slower rate than dog no. 19 and showed many effects of the copper deficiency, such as poor coat, frequent anorexia, and lack of body fat. The skeletal growth of the two dogs, however, was practically identical.

DISCUSSION

The individual phases of iron metabolism such as absorption, transport, storage and utilization are probably all conditioned to a large extent by the nutritive state of the animal. The extremely efficient use of small amounts of dietary iron shown by our dogs on milk diets suggests that under these conditions all phases of iron metabolism proceed in excellent harmony. Although copper did not appear to be essential to absorption or storage of iron, it was shown to be absolutely essential to utilization of iron to build hemoglobin.

Hutchison ('38) clearly showed recently that copper enhances the ability of the human infant to utilize stored iron. Barer and Fowler ('37), Maurer, Greengard, Curtis and Kluver ('34), and Josephs ('39) have demonstrated the effect of copper in increasing utilization of iron in human infants. Josephs ('39) recently drew attention to the phenomenon previously observed by Reimann, Fritsch and Schick ('37), and Brock ('37) that the hemoglobin response often ceases in anemic infants even though sufficient iron has been given to warrant an increase to 100%. In view of our work we would expect this to occur if copper were not supplied with iron. Josephs further points out that "in the first 6 months of life when the conditions are such that relatively rapid hemoglobin formation takes place, iron of the stores is thoroughly utilized." This may be explained by the fact that the period of early infancy is characterized by both high copper and high iron stores and that copper depletion, or dilution in the tissues, must parallel that of iron. Frost, Potter, Elvehjem and Hart ('40) have recently shown the need for copper with iron in overcoming hemorrhagic anemia in dogs on milk diets. The addition of copper wherever iron therapy is indicated seems no less logical to us than the addi-

tion of the iron itself since only in the presence of adequate copper is iron useful.

In their recent studies using radio-active iron, Hahn, Bale, Lawrence and Whipple ('39) obtained evidence showing that the extent of iron absorption is proportional to the need for it. Our work lends strong support to this idea and further shows that iron absorption and utilization may approach 100% under well-defined conditions of need. We cannot refrain from again pointing out that the ration used by these workers, as they have so often demonstrated, does not provide their experimental animals with optimal amounts of those many factors which are so clearly needed to allow an optimal rate of blood building.

Some degree of caution should be urged regarding the use of cobalt as a hematopoietic agent. Although many instances in which small amounts of cobalt have appeared to stimulate hematopoiesis in dogs on milk diets are on record in this laboratory, a clear-cut need for this element has not yet been established. We have found it actually to inhibit hematopoiesis under certain conditions of anemia (Frost, Elvehjem and Hart, '39) and have also succeeded in producing polycythemia under still different conditions. Experiments on the need for cobalt and its polycythemia producing action are now being made.

The very small amount of dietary iron needed by anemic puppies on milk diets both to overcome their anemia and to grow normally is noteworthy. This is especially true when one considers the massive doses of iron used in therapy of so-called iron-deficiency anemia in human infants. That large amounts of iron are more effective than small doses in curing anemia has been recognized for some time (Heath, '33; Witts, '33), but why this should be the case is not at all apparent. In this regard the importance of the general nutritive state cannot be over emphasized. It is readily apparent that infants subsisting largely on milk are prone to develop not only a deficiency of iron, but of copper as well. Another case in hand which suggests the inadequacy of iron alone in treatment of all secondary anemias is that of vitamin B_6 anemia in dogs

(Fouts, Helmer, Lepkovsky and Jukes, '38). McKibbin, Black, Madden and Elvehjem ('39) have produced severe anemia in dogs by the use of a vitamin B_6 deficient ration which supplies adequate iron. The anemia was cured by addition of the crystalline vitamin alone.

Although Hahn ('37) and Heath and Patek ('37) in their recent reviews on iron metabolism minimize the importance of copper as an adjunct to iron in hematopoiesis, they offer no workable solution to the question of massive iron dosage. Other deficiencies than that of copper may operate to prevent or hinder utilization of dietary iron, but the probability of copper deficiency as the limiting factor in many cases of hypochromic anemia still demands clinical attention. Consideration of all the factors clearly involved is most certainly required in any investigation or treatment of anemia.

CONCLUSIONS

1. Ten milligrams of soluble inorganic iron per dog per day are somewhat in excess of the amount needed by dogs growing at the rate of 100 gm. per day. Two milligrams of copper per day proved adequate. The ratio of requirement of iron to copper is only about 5 to 1.

2. Even when supplied in small amounts, iron may be stored. This stored iron becomes available for hemoglobin building only in the presence of adequate copper.

3. The per cent of iron utilization for blood hemoglobin building will depend upon the dietary supply of all factors concerned in hematopoietic activity. When only the theoretical requirement of iron is fed to dogs on iron-free, but otherwise adequate diets, utilization of that iron will approach 100%.

4. Further evidence has been obtained for the importance of cobalt as an adjunct to copper and iron for maximal hematopoiesis in dogs on milk diets.

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THE ANTI GREY HAIR VITAMIN, A NEW FACTOR IN THE VITAMIN B COMPLEX [1]

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SEVEN FIGURES

When black and piebald rats were fed several weeks on certain artificial diets deficient in certain factors of the vitamin B complex, we observed that the black hair turned grey (Lunde and Kringstad, '38). This phenomenon was especially pronounced with diets rich in vitamin B_6 and poor in the so-called "filtrate factor." This greying of the fur of black rats was also observed some years ago by Bakke, Aschehoug and Zbinden ('30) when using a certain vitamin B-free diet and wheat germ as a source of vitamin B. These authors were of the opinion that this phenomenon was caused by the presence of some poisonous compound in the wheat germ rather than by the lack of some new factor of the vitamin B complex. Morgan, Cook and Davison ('38) simultaneously with us announced that they had observed greying of black rats on diets poor in the filtrate factor. These authors were of the opinion that the greying of the fur was due to lack of the "filtrate factor." Our experiments, however, indicate that there is a new vitamin in the B complex distinct from the growth-promoting factor present in the "filtrate" fraction.

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[1] Read at the 98th Meeting of the American Chemical Society in Boston, Mass., September, 1939.

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It is generally recognized that the vitamin B complex contains B_1 or thiamin, B_2 or riboflavin, nicotinic acid or pellagra-preventive factor, B_6 recently demonstrated to be 2-methyl-3-hydroxy-4,5-di(hydroxymethyl) pyridine, "filtrate factor" or chick antidermatitis substance which appears to be identical with pantothenic acid, and the rat growth "factor W" of Elvehjem and associates (Elvehjem, Koehn and Oleson, '36; Frost and Elvehjem, '37, '39; Oleson, Bird et al., '39) which may be separated from the "filtrate factor" by precipitation with alcohol and ether. Several authors have used the term "filtrate factor" to mean the growth-promoting factor or factors remaining in the solution after treatment with fuller's earth intended to remove B_6. This, however, is rather unfortunate, because the filtrate probably contains several distinct factors. For example, the chick anti-dermatitis factor (pantothenic acid) is present as well as the rat growth factor W. According to Woolley et al. ('38), this chick antidermatitis factor can be extracted with ether in neutral solution. We have been able to show that the rat growth factor contained in the filtrate after treatment of a yeast extract with Fuller's earth is not extracted with ether under these conditions (Kringstad and Lunde, '39). This indicates that our rat growth factor is distinct from the chick antidermatitis factor. In order to define it more clearly, we prefer to designate the rat growth factor in the filtrate as B_w.

GROWTH-PROMOTING FILTRATE FACTOR B_w

When an extract of fish liver is treated with fuller's earth, vitamin B_6 is adsorbed. We have been able to show that the filtrate thus freed from B_6 has growth-promoting properties when given to rats subsisting on a diet containing sufficient amounts of thiamin, riboflavin, nicotinic acid and B_6 (Lunde and Kringstad, '38). This result is in accordance with the observations made by Lepkovsky, Jukes and Krause ('36) working with liver, and by Edgar and Macrae ('37) working with yeast. These results, however, have not always been verified by other investigators. We have found that this may

be due to the fact that the pH of the yeast extract greatly influences the adsorption on fuller's earth (Kringstad and Lunde, '39). At pH 5-6 we were able to demonstrate that B_6 even after three adsorptions is not quantitatively removed from the extract. At pH 1.0 factors other than B_6 are also adsorbed. At such a strongly acid reaction and under our experimental conditions, compounds are also formed which are poisonous to the avitaminotic rats. In view of the above experiments adsorption on fuller's earth does not appear to be suited for achieving a distinct separation of vitamin B_6 from the growth-promoting factor in the filtrate of a yeast extract.

Another possibility for securing this desired separation is to precipitate the B_6 by means of phosphotungstic acid. Our experiments indicate that by this procedure one can remove B_6 quantitatively from the solution.

The amount of factor B_w in certain foodstuffs was determined. Rats were allowed to subsist on an artificial diet containing sufficient amounts of thiamin, riboflavin, nicotinic acid and B_6. When growth had ceased, evidenced by mere maintenance of body weight, a test quantity of the food in question was fed daily (fig. 1). The amount of factor B_w necessary to produce an average increase in weight of 7 gm. per week was taken as defining the rat-growth unit of this factor (table 1). Cane sugar was the carbohydrate used in the basal diet. The protein source was fish meal with a low ash content. This fish meal contains only insignificant traces of the factor B_w, and it is at the same time a good source of vitamin B_6. Peanut oil was the fat used in the diet. Cod liver oil provided vitamins A and D. These experiments showed that of the substances tested the best sources of the rat-growth factor B_w were liver and yeast. Wheat germ, corn and other cereals rated as fair. Mammalian liver proved to be the best source containing at least 8 rat units per gram. Therefore the use of liver was adopted for the further experiments designed to yield a suitable concentration of this factor.

A liver concentrate prepared by extraction of a cruder extract with phenol[3] (Laland and Klem, '37) was tested on rats using our vitamin B_w-free diet containing fish meal as the source of protein. A maximum growth was obtained when 0.1 cc. liver concentrate was given daily, corresponding to 5 gm. of the original fresh liver (fig. 2). From this experiment it is evident that the factor B_w is soluble in phenol. The growth factor W of Elvehjem et al. is also soluble in phenol (Frost and Elvehjem, '39), and it is possible that these two factors
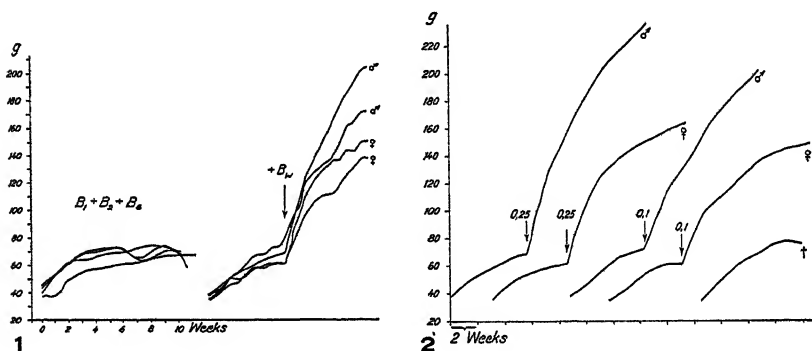
Fig. 1 Growth curves of rats subsisting on a vitamin B-complex free supplemented with B_1, B_2 and B_6 as indicated. The right-hand group of curves indicate the growth performances of rats which received in addition a supplement containing the B_w growth factor.

Fig. 2 Growth curves of rats fed the same basal diet and vitamin supplements as the animals in figure 1. When growth had decreased markedly, the rats received 0.25 or 0.1 cc., of the phenol soluble fraction of the liver extract. It is evident that this fraction contained the growth factor B_w.

are identical. In a new experiment phosphotungstic acid was added to the phenol-soluble liver concentrate. The resulting precipitate was filtered off and the excess of phosphotungstic acid removed by barium hydroxide. The barium precipitate was removed by centrifugation, and the excess of barium precipitated by means of sulfuric acid. The barium sulfate was again removed by centrifuging, and the clear solution evap-

[3] "Pernami forte" prepared by Nyegaard and Company, Oslo.

orated in vacuum to the original concentration. When rats were given 0.25 cc. of this solution, they exhibited a growth corresponding almost to that obtained when the original liver concentrate was used. This experiment shows that the factor B_w is not precipitated by phosphotungstic acid.

The growth factor B_w is almost quantitatively dissolved in an acetone-water solution. This is shown by several repetitions of the following experiment with the same result. A phenol soluble liver extract was evaporated in vacuum until of a syrupy consistency, and this syrup repeatedly extracted

TABLE 1

Distribution of the rat growth factor B_w in certain foods

FOOD	UNITS PER GRAM	FOOD	UNITS PER GRAM
Fish products		Mammalian tissue	
Fish liver meal	2.8	Liver: ox	6.0
Cod roe, canned	2.0	Liver: pork	4.0
Cod liver	1.4	Pork	0.8
Sardines, brisling, canned	0.9	Beef	0.8
Sardines, herring, canned	0.9	Miscellaneous	
Herrings, kippered, canned	0.7	Yeast: brewery	6.0
Plaice, flesh	0.6	Yeast: bakery	3.0
Cod milt	0.5	Wheat germ	2.0
Herring milt	0.5	Corn	0.7
Cod, flesh	0.2	Milk, cow's	0.15

at ordinary temperature with acetone containing 10% of water. The acetone extracts were evaporated in vacuum until syrupy in consistency and this material then dissolved in sufficient water to give the original concentration. Tested on rats fed the vitamin B_w-free diet, ¼ cc. of this solution corresponding to about 10 gm. of fresh liver gave maximum growth. The part of the liver extract not dissolved in acetone-water was also tested on rats. The rats received an amount corresponding to 10 gm. of fresh liver and showed only a very poor growth amounting to an average of about 6 gm. per week (fig. 3).

The growth factor B_w is not precipitated with mercuric chloride. In support of this statement the following experiments may be cited. The acetone soluble part was evaporated until syrupy in consistency. The syrup was dissolved in methyl alcohol and a solution of mercuric chloride in methyl alcohol added. The precipitate was removed by centrifuging, emulsified in water slightly acidified with sulfuric acid, and the mercury removed by means of hydrogen sulfide. The excess of H_2S was removed by carbon dioxide. The remaining material was then evaporated and tested on rats. It proved to have no growth-promoting properties. On the other hand, the
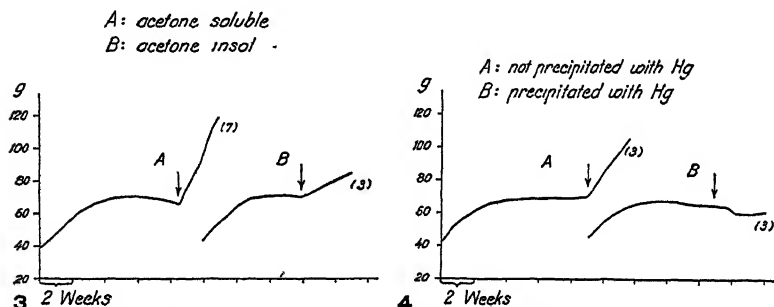
Fig. 3 Average growth curve of rats fed a B_w free diet and in addition acetone-soluble (A) or acetone-insoluble (B) part of liver extract, respectively. The figures in parentheses indicate the number of rats on the experiment.

Fig. 4 Average growth curves of rats on a B_w free diet, and receiving in addition either the filtrate after the precipitation with Hg (A) or the part of the liver extract precipitated by Hg (B). Note that the growth factor is not precipitated by treatment with Hg.

filtrate, after the mercury precipitation, was treated with H_2S to remove the mercury, and the sulfide filtered off. This filtrate was evaporated in vacuum, dissolved in water and tested on rats. Twenty-five hundredths cubic centimeters of the solution, corresponding to about 25 gm. of the original fresh liver, gave optimal growth (fig. 4).

The growth factor B_w is evidently unstable toward heat. Our original phenol soluble liver extract was heated in an autoclave for 8 hours at 120° C., and then tested on rats.

Twenty-five hundredths cubic centimeters of this autoclaved liver extract, corresponding to about 10 gm. of fresh liver, gave very poor growth. The weekly increase in body weight was on an average only 5 gm. (fig. 5).

THE ANTI GREY HAIR FACTOR B_x

When using certain diets rich in B_6 and poor in the filtrate factor (Lunde and Kringstad, '39 a), we observed a hemorrhagic condition especially on the paws, and similar to that also described by Elvehjem et al. ('37) and György et al. ('37). In addition, a striking fact was observed. In all our
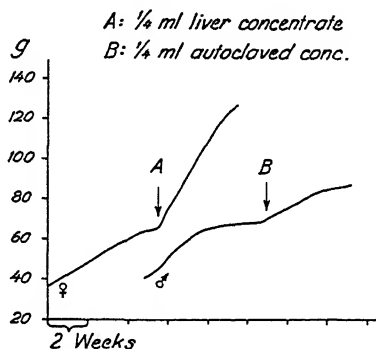
Fig. 5 Average growth curves of rats showing the adverse effect of autoclaving on vitamin B_w.

feeding tests we had been using piebald rats. We observed a peculiar greying of the black hair of these animals (fig. 6). In view of the evidence cited below, we are of the opinion that this greying of the black fur of the rats is due to lack of a new vitamin distinct from the growth-promoting factor B_w in the so-called filtrate fraction. We have provisionally designated this vitamin B_x.

In our first experiments with B_6 this syndrome was always observed when fish meal was used to cure the rat dermatitis developed by subsistence on the B_6-deficient diet. Later, several different diets were tested and it was observed that a

diet with fish meal as the source of protein gave the most pronounced greying of the hair. When using wheat germ in the diet, we encountered many cases where no greying of hair occurred, or the phenomenon was present but in a very limited degree. This was difficult to understand, in view of the fact that this greying phenomenon was first observed by Bakke et al. ('30), when using this diet. Morgan and associates
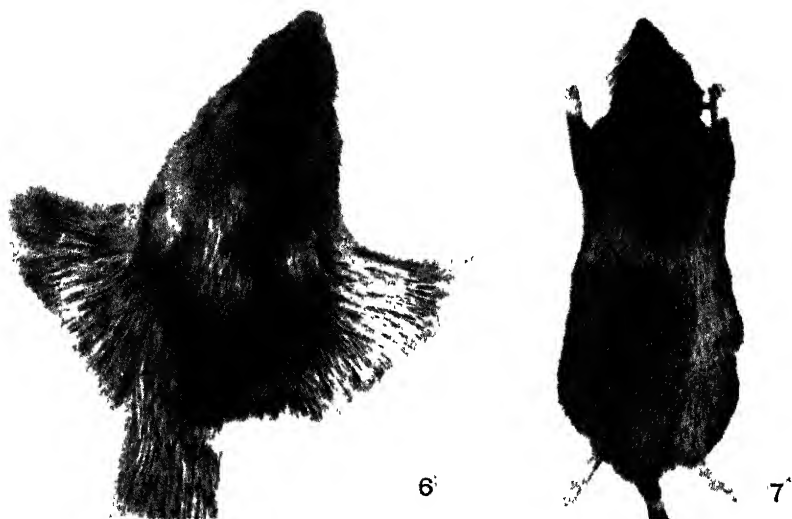
Fig. 6   Piebald rat. Note the black fur changed to grey in color. Black spots of original color are still evident.

Fig. 7   Black rat which has developed greying of the fur chiefly over the body and to a lesser extent, on the head.

('38) also reported that the greying was especially pronounced when wheat germ was used.

In all further studies black rats were used. It was noticed that the greying of the hair is always symmetrical. It is usually first observed on the head between the ears and at a certain spot on the back. In some cases it also starts on both sides of the body but always symmetrically (fig. 7). The color is not a dull grey but a more silvery grey. In some cases the

whole rat becomes silver-grey in appearance. The avita-minosis can be cured with products containing the new factor, for example, yeast and liver. Mammalian liver is especially rich in the new factor, but it is also present in fish liver.

The greying of the fur is not related to the anaemic greying of hair associated with lack of iron or copper salts. The administration to grey rats of small amounts of iron, copper and manganese salts is without effect (Lunde and Kringstad, '39 a). The new factor B_x cannot be identical with the growth factor B_w. We have observed that rats show the greying of the fur when subsisting on certain diets whether they exhibit growth or not.

The new anti-grey-hair factor B_x seems to be more heat labile than the growth factor B_w. In support of this statement, we may cite an experiment in which a yeast extract was heated for 3 hours at $100°C$., concentrated in vacuum over a water bath, and then tested for both of these factors. The anti-greying substance had been lost whereas the growth factor B_w was still present in considerable amount.

Factor B_x proves to be soluble in phenol. As mammalian liver proved to be one of the best sources of the anti-grey-hair substance, our phenol soluble liver extract was tested on rats for its content of B_x. The B-free diet was supplemented with ample amounts of thiamin, riboflavin, nicotinic acid and a B_6 concentrate. After 8 to 10 weeks, the rats showed the typical greying of the black fur. The animals were then given 0.25 cc. of the liver concentrate corresponding to about 10 gm. of fresh liver. The rats immediately exhibited good growth and the fur changed to black in color within a period of 6 weeks.

The above experiment was repeated with rats allowed to subsist upon our fish-protein diet. After from 8 to 10 weeks, the rats were silvery grey in color, at which time they were given our liver concentrate as a dietary supplement. This was promptly followed by excellent growth of maximum degree. After 3 weeks the fur began to darken and after 7 weeks it had recovered its normal black color. The first change was observed on the head where the animal's grey hair was re-

placed by new black hair. As was stated previously, the greying was always symmetrical (figs. 6 and 7).

SUMMARY

From these experiments it appears evident that in addition to the known members of the vitamin B complex, the rat also needs two other factors, one a growth factor designated B_w which may be identical with the "factor W" of Elvehjem et al., the other a substance B_x required to prevent and to cure the greying of black hair.

Factor B_w is soluble in phenol but not in ether in acid solution, a fact which distinguishes it from the chick anti-dermatitis factor. B_w is easily soluble in acetone and water. It is not precipitated either by phosphotungstic acid or mercuric acetate. This factor is destroyed by autoclaving for 8 hours at 120°C. If yeast is heated for 3 hours at 100°C., a considerable amount of B_w is still present. Liver and yeast are excellent sources of this substance.

Factor B_x is likewise present in liver and yeast in large amounts. It is soluble in phenol but is not precipitated by phosphotungstic acid. This anti-grey-hair factor seems to be more heat labile than the growth factor B_w, evidenced by the fact that it is completely destroyed in yeast by heating for only 3 hours at 100°C. That the growth factor and the anti-grey-hair factor are distinct entities is further supported by the fact that they show differences in their respective distributions in the foods tested.

After this paper was read before the American Chemical Society, there appeared the communication by Oleson, Elvehjem and Hart ('39) confirming us. They conclude that this factor "is distinct from all factors of the vitamin B complex which have thus far been identified and associated with specific function in the nutrition of the rat. Further, the factor does not seem to be involved in the growth of the rat."

We also wish to add that we in new experiments have been able to show that a substance with growth-promoting properties, when tested on rats fed a B_w free diet, can be extracted

with ether from an acidified liver extract (Lunde, '39). This fact seems to indicate that we are dealing with a new growth-promoting "filtrate" factor distinct from B_w, unless the B_w-factor should be present in different forms in yeast and in liver.

We have also been able to show that the anti-grey-hair vitamin B_x is necessary for the normal development of the pelt of silver foxes (Lunde and Kringstad, '39 b).

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CYSTINE AND METHIONINE DEFICIENCY
IN MOLD PROTEINS[1]

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NINE FIGURES

Little attention has been given to the nutritive value of microbial proteins, due partly, perhaps, to the difficulty of obtaining large amounts of microbial material, and partly to the fact that although many foods contain microorganisms in large numbers, still the microbial substances are small in comparison to the amounts of the food itself. This is true even in such foods as sour milk, soya sauce, sauerkraut, various kinds of cheese, etc., which contain many bacteria or molds. These organisms, dead or alive, are being consumed, however, and their food value should be known. Then, too, the ease of converting inorganic nitrogen to microbial proteins with cheap carbohydrates such as straw, sawdust, or other plant residues as energy materials, has suggested the possibility of feeding domestic animals or human beings these proteins. This was suggested, more or less fancifully, by Robertson ('20), but has actually been done by Pringsheim and Lichtenstein ('20), who fed to animals straw fortified with inorganic nitrogen fertilizer on which Aspergillus fumigatus had grown.

It is well established that many molds and bacteria can synthesize their own proteins from inorganic nitrogen. The frequently quoted work of Abderhalden and Rona ('05), who

found that Aspergillus niger was able to synthesize straight-chain amino acids has been extended by Vorbrodt ('19, '34), Takata ('29a, b), Skinner ('34), and others, so that we know now that tyrosine, tryptophane, phenylalanine, proline, and many other amino acids are also readily formed from inorganic nitrogen by molds.

Actual feeding experiments with molds grown in inorganic nitrogen-containing media have rarely been carried out. Takata ('29a, b) grew Aspergillus oryzae in a medium with dextrin as a source of energy and $(NH_4)_2SO_4$ as the sole source of N. The mold was harvested, and fed to rats at 11, 15, and 19% protein levels in otherwise complete diets. A very slight growth on an 11% protein diet, better on a 15% diet, and a good growth on a 19% protein ration resulted. After 2 months the rats on the 19% diet no longer gained weight and were not benefited by the addition of cystine to the diet, although the protein by analysis contained only traces of cystine. The small number of animals used and the fact that the cystine was not added until late in the course of the feeding experiment left the results rather inconclusive as far as the cystine deficiency of this particular mold was concerned. The results, however, indicate that this mold may serve at least as a partial source of protein for rats. Takata considered the digestibility of the proteins to be good from the experiments which he carried out.

One of us (Skinner, '34) and Skinner (J. T.), Peterson and Steenbock ('33) independently studied the value of molds as a source of protein for animals. The few feeding experiments carried out by the former investigator were only incidental for demonstrating the synthesis of aromatic amino acids by molds. It was found that Penicillium flavo-glaucum, the mycelial growth of which was fed at a 9% protein (29% mold) level in an otherwise complete diet, supported only poor growth. A much better but still not a normally good growth was obtained at an 18% protein level, while a 9% mold protein together with either a 9% casein or a 9% Zea mays protein (20% corn-gluten meal) diet supported a normal growth

for several weeks, although each of these proteins alone is known to be deficient. It was further shown by paired-feeding experiments that cystine was the first limiting amino acid of the mold. The statistical values rather than the data were published for this last set of experiments. Skinner, Peterson and Steenbock ('33) and Gorcica, Peterson and Steenbock ('35) also showed that molds were very poor sources of proteins but could be used in conjunction with other deficient proteins to support a good growth. These authors did not find that the addition of cystine increased the value of the proteins when added to the diet either at the beginning or later in the experiment.

Several workers, from Sunderlin and Werkman ('28) on, have studied the vitamin synthesis by molds. In general it has been found that the vitamin B complex is synthesized by molds, but considerable quantities, often more than the animals willingly eat, have to be consumed to supply optimum quantities of the B complex.

There are several points on the nutritive value of mold proteins that remain to be studied. The present report will be confined to the sulfur-containing amino acid deficiencies of a few of the common molds.

EXPERIMENTAL

The molds were grown and prepared as reported previously (Skinner, '34). The medium used varied with the species[3], as

[3] Penicillium flavo-glaucum, isolated from air; Aspergillus oryzae, secured from the Department of Agricultural Bacteriology, University of Wisconsin: $Ca(NO_3)_2\cdot 4H_2O$, 2 gm.; KH_2PO_4, 2 gm.; $MgSO_4\cdot 7 H_2O$, 1 gm.; $(NH_4)_2SO_4$, 2 gm.; $FeCl_3$, trace; glucose, 20 gm.; H_2O, 1000 cc.

Penicillium roqueforti, secured from the Department of Agricultural Bacteriology, University of Wisconsin; Penicillium species 1 and 2 isolated from soil, unidentified: $(NH_4)_2HPO_4$, 2 gm.; $(NH_4)_2SO_4$, 1 gm.; $MgSO_4\cdot 7 H_2O$, 0.5 gm.; $CaCl_2$, 0.1 gm.; KCl, 0.5 gm.; $FeCl_3$, trace; sucrose, 30 gm.; H_2O, 1000 cc.

Aspergillus nidulans, from Dr. A. T. Henrici, University of Minnesota: $(NH_4)_2HPO_4$, 2 gm.; K_2HPO_4, 1 gm.; $NH_4H_2PO_4$, 1 gm.; $(NH_4)_2SO_4$, 1 gm.; $MgSO_4\cdot 7H_2O$, 0.5 gm.; $CaCl_2$, 0.1 gm.; $FeCl_3$, trace; sucrose, 30 gm.; H_2O, 1000 cc.

Geotrichum lactis (often known as Oidium or Oöspora lactis) isolated from sour milk: $(NH_4)_2SO_4$, 1 gm.; $(NH_4)_2HPO_4$, 2 gm.; $NH_4H_2PO_4$, 1 gm.; K_2HPO_4, 1 gm.; $MgSO_4\cdot 7H_2O$, 0.5 gm.; $CaCl_2$ 0.1 gm.; $FeCl_3$, trace; glucose, 30 gm.; H_2O, 1000 cc.

more growth could be obtained by changing the medium slightly for the different species. In each medium, the only organic compound was either glucose, U.S.P., or cane sugar. The N source was either ammonium salts or ammonium salts and nitrates. These and the other inorganic salts used were C.P. grade. No addition was made of zinc or other salt known to be stimulating to molds in minute quantities. Obviously the traces of these salts considered necessary to molds were obtainable as impurities in the salts, the distilled water used, or the 1-liter medicine bottles used as incubation flasks, since a heavy growth was secured.

The resulting dried ground mold which had been heated for a short time at 100° C. was analyzed for total N and the protein content was estimated by use of the factor 6.25 after allowing for the nitrate and NH_3 nitrogen which remained in the mold from the medium in which the mold had grown. This factor is probably not correct as some of the mold N is undoubtedly nonprotein in nature. The rations were made with a 30% mold content in the diet, which brought the "protein" content to about 9-10%, depending upon the mold. The rest of the diet consisted of 15% fat[4], 5% salts (McCollum's 185 with iodized salts substituted for NaCl) and cornstarch. All ingredients were ground powder fine and were thoroughly mixed. Each rat received daily 2 drops of U. S. P. cod liver oil and 100 mg. of a vitamin B complex concentrate[5] in the form of pills. With some molds, after the animals had been on the diet for 30 days, 0.8 gm. dried yeast was given twice a week.

The rats were used in pairs, following the technique of Mitchell and Beadles ('30). They were litter mates of the same sex, and each pair, as closely alike as possible in weight, was put on the diet at the age of 21-28 days. One member of each pair received the ration to which was added 0.25% cystine or methionine, and the other member a similar ration with either 0.25% alanine or an equivalent amount of mold.

[4] Crisco, a vegetable fat.
[5] Harris vitamin B complex.

The amount of food each animal consumed was measured daily. The food wasted was sieved to separate the feces, and this was allowed for. Each day the animal which was willing to eat the most food was given the amount the other member of the pair had eaten the previous day, while the one which ate the lesser amount was allowed to eat ad lib. In this way the animals were truly paired, not only as to initial weight and sex, but as to food intake as well. The animals were weighed to the nearest $\frac{1}{2}$ gm. every 5 days. After the first period or so, the rat with the best appetite was invariably the one which was getting the cystine or methionine diet.

If the member of each pair which was fed a sulfur-containing amino acid regularly gains more weight than the other, it may be concluded that the amino acid is beneficial. To test whether the apparent superiority of the cystine or methionine diets is real, one can either look at the charts and estimate whether the results are significant, an all too common method which is sufficient only if the data are outstanding, or one can subject the results to statistical analysis and thus ascertain objectively the chances that the results might be fortuitous. Of the three statistical methods suggested by Mitchell and Beadles ('30), only one was used.

Given the number of weighings of paired animals for each particular mold (n) and the number of times the cystine or methionine fed animal gained more than its control (a), one can calculate the index suggested by Mitchell and Beadles:

$$\frac{a-\frac{n}{2}}{\sqrt{\frac{1}{2} \times \frac{1}{2} \times n}}$$

From this, using the tables of Pearson ('31), or other tables derived from them, one can find the probability (P) that chance rather than the diet caused the one member of the pair to gain more often than the other. The above formula (the number of times that the standard deviation of the frequency distribution is exceeded by the divergence between 1/2 of the number of feedings and the number of times that the cystine

or methionine-fed animal gained more than the control) may be simplified to:

$$\frac{2a-n}{\sqrt{n}}$$

It will be noted that all our results show P to be less than 0.05. It is usually considered that any value of $P = 0.05$ or less indicates that the results are significant, and any value of $P = 0.01$ or less is considered highly significant. Here the P value may be interpreted as the probability that chance rather than differences in diets caused the cystine or methionine fed animals to gain in weight more often than the controls.

The graphs show the growth curves, the amount of food consumed, and the values of P. In all cases, it will be seen that the cystine or methionine fed animals gained significantly

Aspergillus nidulans
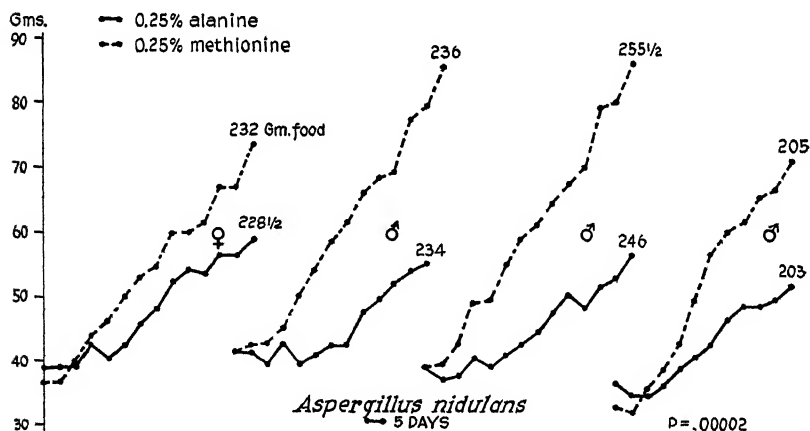
more than the others except in the case of one of the unidentified Penicillium species. Here no growth resulted without cystine. These animals consumed progressively less and less food, and therefore after they had decreased in weight until there was evident danger of death, the cystine ration was given to all animals ad lib. A slight gain resulted in most cases. Here the evidence is less clear-cut that the mold was deficient in sulfur-containing amino acids, although it indicates that this may well be true. Methionine was used in place of cystine

with only some of the molds, since the work was in progress
before the appearance of Rose's ('37) paper, wherein it is
shown that methionine rather than cystine is essential, but
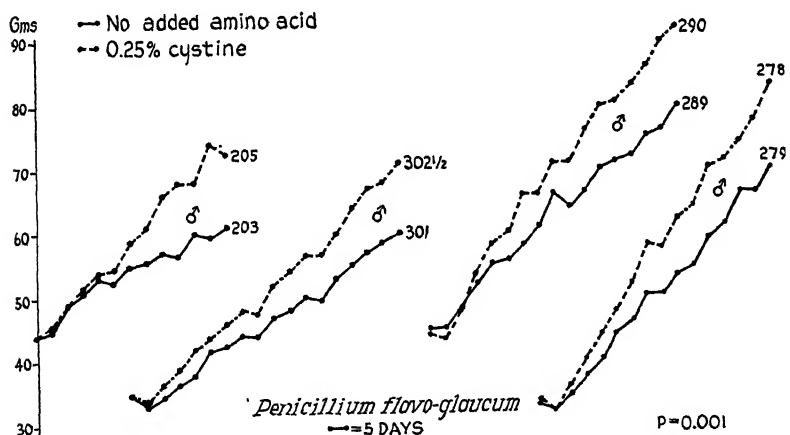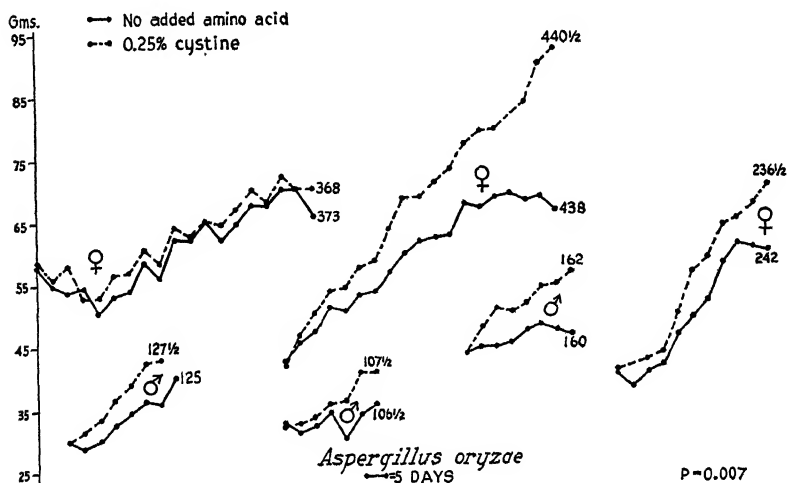
that cystine can replace methionine after the minimal require-
ments of this amino acid are met. The evident response to
cystine showed that the molds were not entirely devoid of
methionine, but were deficient in sulfur-containing amino

acids. From our results, however, it is quite possible that methionine would have demonstrated greater response than cystine did.

The slow growth of animals is probably partly due to the fact that the molds are not palatable. Results not reported here show that animals differ markedly in their appetites for
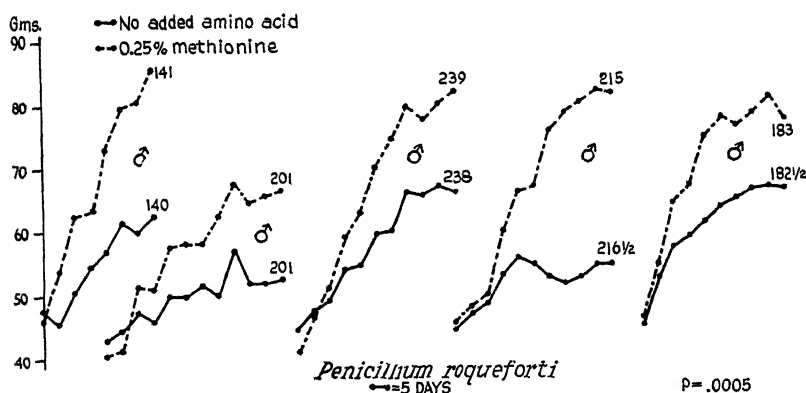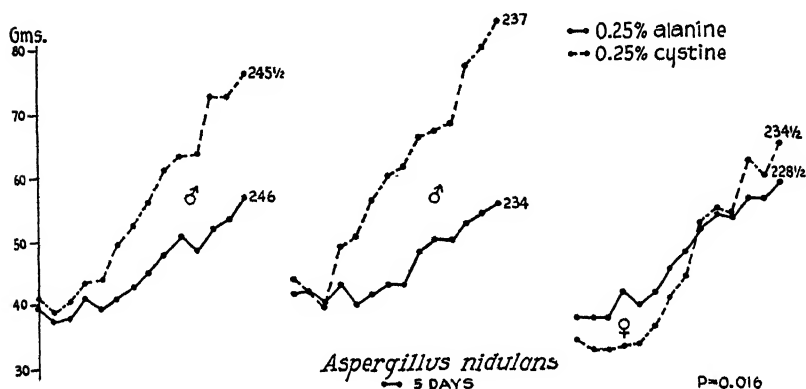
foods containing about 30% mold. However, after the apparent distaste for the mold was at least partially overcome, the animal grew fairly well on a complete diet containing mold material. One female rat fed after 28 days of age on a ration consisting of casein 15 gm., salts 5 gm., Penicillium roqueforti 30 gm. (9% protein), fat 15 gm., starch 32 gm., agar-

agar 3 gm., with the usual daily dose of vitamins, grew slowly
at first, but at the age of 148 days weighed 175 gm. She was
then placed in a cage with a male and became pregnant. Two
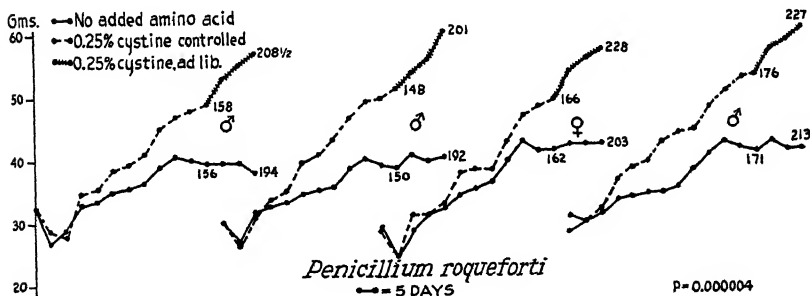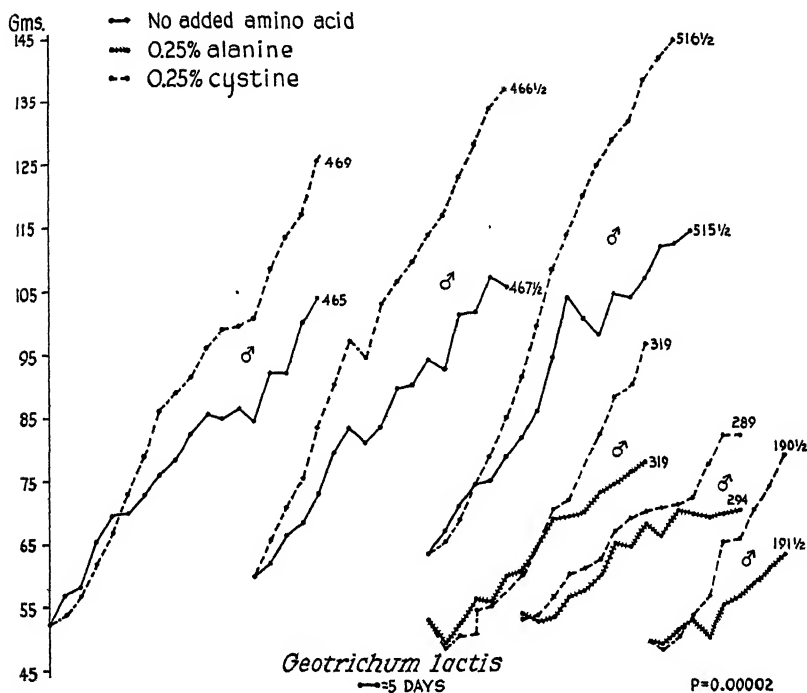
days before her litter of eight was born, when she was 173
days old, she weighed 214 gm. Other rats fed on the same diet
with the same mold (which the graphs show to be one of the

poorest) did not do so well, but in every case the growth was correlated with the amount of food consumed. It is obvious that the mold was not toxic. This phase of the work will be discussed further in a later paper. All of the molds, with the
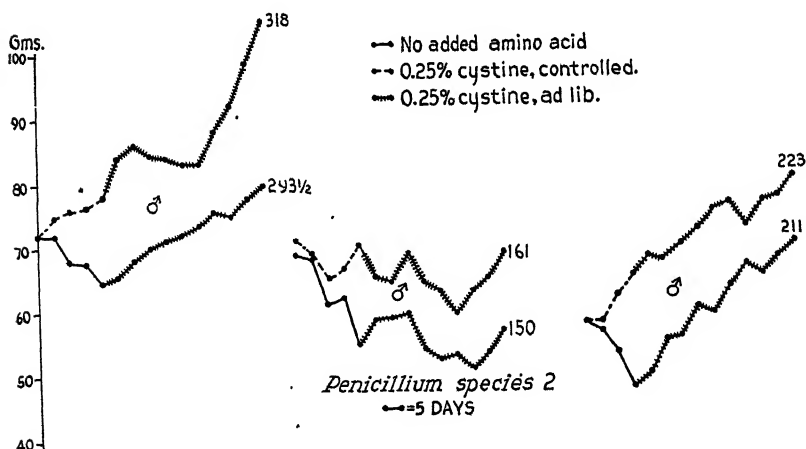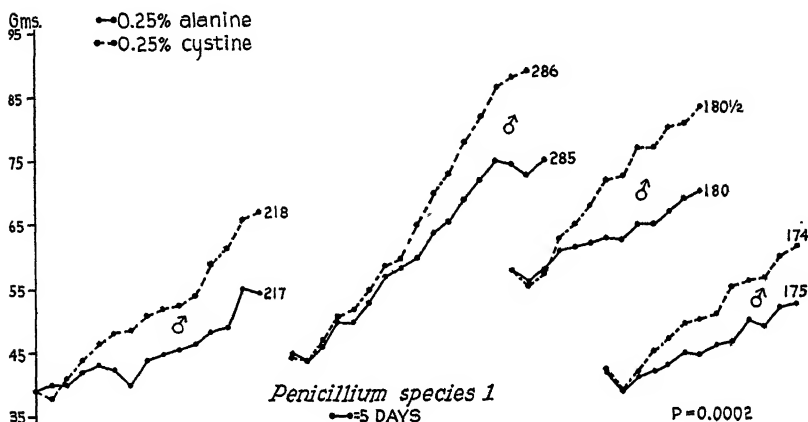
exception of Aspergillus nidulans, were tasted by the authors and found to be extremely bitter. Geotrichum lactis, which was most readily eaten by the animals, was noticeably less bitter than the others.

It should be noted that after the first week or two the greater gain in weight of the cystine or methionine fed animals occurred with animals partly starved and that the gain over the control mates continued after the control weighed considerably less; that is, the food containing cystine or methionine supported greater growth than the same amount of food without the sulfur-containing amino acid, even though the animals making the greater growth were already larger and needed more food to maintain their weight, and were willing to eat more than they got. Correcting for this difference in weight would merely show a greater spread in the graphs between the two members of a pair, and make the results appear even more significant.

It is evident that the molds studied were deficient in sulfur-containing amino acids. Whether this is true of all species of molds grown under all conditions is not known. The work of Gorcica, Peterson and Steenbock ('35) would indicate that it is not true. Our methods differed from theirs with respect to species, media, harvesting, and diets, especially in that yeast and vitamin B concentrate were added. But, with the exception of one of the Penicillium species, where the data are suggestive rather than definite, the molds which were studied by us were deficient in sulfur-containing amino acids under the conditions of our experiment.

SUMMARY

The molds, Aspergillus nidulans, A. oryzae, Geotrichum lactis, Penicillium flavo-glaucum, P. roqueforti, and two unidentified species of Penicillium were fed to rats as a sole source of protein, in diets low in protein. It was found that the addition of 0.25% cystine or methionine greatly enhanced the growth of rats over those having 0.25% alanine or an equivalent amount of mold added to the diet. It is concluded that the sulfur-containing amino acids are in too low concentration in mold proteins to promote good growth.

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THE CURE OF NUTRITIONAL MUSCULAR DYSTRO-
PHY IN THE RABBIT BY ALPHA-TOCOPHEROL
AND ITS EFFECT ON CREATINE METABOLISM

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FOUR FIGURES

Nutritional muscular dystrophy was first described by
Goettsch and Pappenheimer ('31) in rabbits and guinea pigs.
They were unable to ascribe the disease to a lack of any of
the known vitamins. Morgulis and his co-workers (Morgulis
and Spencer, '36 a; Morgulis et al., '38) reported that at least
two factors, both contained in whole wheat germ, were re-
quired to cure the disease in rabbits: a water-soluble factor
present in defatted germ, and a fat-soluble factor present in
the unsaponifiable matter of the oil.[1] This suggested that
the fat-soluble factor might be vitamin E. Support for the
hypothesis was furnished by Olcott's ('38) observation that
muscle lesions similar to those in dystrophic guinea pigs
occur in the paralyzed suckling young of vitamin E-deficient
female rats. Barrie ('38) and Goettsch and Ritzmann ('39)
have recently shown that this disorder in suckling rats is
prevented by alpha-tocopherol.

On the other hand, considerable evidence existed against
the postulated identity of vitamin E and the fat-soluble anti-
dystrophy factor required by herbivora. The dystrophy-pro-
ducing diet of Goettsch and Pappenheimer ('31) apparently

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[1]Only temporary cures were obtained with these concentrates.

contains a significant amount of vitamin E, since both these workers and Morgulis et al. ('38) found that female rats grown and maintained on it exhibited "initial fertility" in the first and second generations, a phenomenon entirely inconsistent with the complete absence of the vitamin. The explanation that the rabbit requires more E than the rat seemed to be eliminated by the observation of Thomas et al. ('38), quoted in greater detail by Mattill ('38 a), that twelve rabbits on a diet deficient in E bore 137 young in 2 years. Such a performance excludes the possibility of severe dystrophy in these animals.

In addition, the muscle lesions in the suckling and adult paralyzed E-deficient rat are quite possibly secondary to degenerative changes in the central nervous system (Lipshutz, '36; Einarson and Ringsted, '38), while in the herbivora no changes in the nervous system have been detected (Goettsch and Pappenheimer, '31; Rogers et al., '31; Chor and Dolkart, '39). Further complications arose as the result of Mattill's ('38 b) report that the addition of wheat germ oil to a synthetic ration protected rabbits from dystrophy, but that the oil could not be completely replaced by potent vitamin E concentrates.

This problem was clarified by our observation, presented in a preliminary note (Mackenzie and McCollum, '39), that rabbits rendered dystrophic on diet 13 of Goettsch and Pappenheimer ('31), supplemented with defatted wheat germ, are cured by alpha-tocopherol. At that time we referred to the sharp drop in urinary creatine effected by the vitamin. Since then, Shimotori et al. ('39) have announced that alpha-tocopherol prevents muscular dystrophy in guinea pigs reared on a synthetic diet, and Morris ('39) has confirmed our observation that alpha-tocopherol cures dystrophy in rabbits.

The present paper is a detailed report of our curative experiments, together with simple criteria for diagnosing and following the development of the disease, and for detecting within a few days a response to potent supplements. Chief among these criteria is the increased creatine output

noted by Morgulis and Spencer ('36 b) in dystrophic rabbits. Data on both dystrophic and cured animals showing the relation of creatine excretion to creatinine excretion, weight changes, and food consumption are presented in figures 1, 2, 3 and 4.

METHODS

By modifying the dystrophy-producing diet 13 of Goettsch and Pappenheimer [2] through the addition of 10% defatted wheat germ, a source, according to Morgulis et al. ('38) of the water-soluble factor, we have limited our attention to the identity of the fat-soluble essential. Rabbits develop dystrophy just as rapidly on this diet as on unmodified diet 13. The defatted germ was prepared by extracting fresh wheat germ in a continuous extraction apparatus for 24 hours with cold U.S.P. ethyl ether.

Diet 13 was prepared twice a week and kept in a refrigerator. The ether-extracted wheat germ was incorporated at the time of feeding. Fresh diet was supplied the animals every 2 or 3 days.

Each rabbit was housed in an individual all-metal metabolism cage. The weight and food intake were recorded daily, and the rabbit closely examined for signs of dystrophy. Urinary creatine and creatinine were determined by the methods of Folin ('14).

[2] Diet 13.

Rolled oats (Quaker)	355 parts
Wheat bran (Pillsbury)	180 parts
Casein (Merck technical)	75 parts
Lard	80 parts
Cod liver oil (Mead Johnson & Co.)	10 parts
NaCl	10 parts
$CaCO_3$	15 parts

Ten grams of ferric chloride, U.S.P. lump, was taken up in about 125 cc. of ether and a little water, and the solution poured over the above ingredients. The mass was shaken in a closed container and allowed to stand for about half an hour. The contents were emptied upon a tray and the ether allowed to evaporate overnight. Finally there was added:

Skimmed milk powder (Merrell-Soule)   275 parts.

All supplements were fed by mouth from a 1 cc. tuberculin syringe equipped with a long blunt needle. Food was withheld for at least 2 hours before and 3 hours after supplementing to prevent mixing of the supplement and the experimental diet in the stomach. Particular importance was attached to this precaution in view of the finding of Lease et al. ('38) that vitamin A is destroyed when it is mixed with the peroxides of rancid fats in the stomach; and the finding of Weber et al. ('39) that vitamin E is not destroyed when administered separately from rancid fat so as to prevent mixing in the gastrointestinal tract. It was thought that such peroxides might be present in our experimental diet.

Most of the animals were either killed when moribund, or found within an hour after death. Autopsies were performed on these animals, and blocks routinely taken from the muscles of both thighs for microscopic examination.

EXPERIMENTAL

Since we have been unable to find a description in the literature of the behavior of dystrophic rabbits, our observations will be presented with the other diagnostic criteria employed, namely, growth, food consumption and creatine excretion.

The deficiency state. Eighteen male and female rabbits, averaging 385 gm. (270 to 470) in weight, gained on the average 220 gm. per week during the first 2 weeks on the experimental diet. The rate of gain declined during the third week, and by the fourth week had fallen to about 100 gm. The maximum weight was attained in 31 days (18 to 42), and represented an average total gain of 740 gm. During the period of gain, food consumption averaged 50 gm. per day. The disease itself has been divided into three stages.

Stage I. The initial stage of dystrophy was characterized by a rise in the daily creatine output from a normal level of less than 10 mg. to over 20 mg. This rise occurred from 18 days before to 1 day after the attainment of maximum weight. The average was 4 days prior to this time. Creatinine excretion did not increase. Following the maximum weight

there was an immediate but gradual decline in thirteen animals, and in the remainder a period of stationary weight of 5 to 12 days' duration. In either case the urinary creatine continued to rise. When the animals ceased gaining, food intake declined abruptly to an average of 33 gm. per day. The average duration of this stage, from the time of maximum weight, was 7 days.

Stage II. This stage was marked by the appearance of the first physical symptoms. The front feet were placed in the food or water jars, the front legs held stiff, and the head slightly retracted. This position was maintained for hours at a time. Several animals did not use the jars when assuming this position, but placed the fore feet well under the body between the hind legs. The second change in behavior (the first change in some cases where the above symptoms were not noted) was the ease with which the rabbits could be laid on their sides, and their slowness in righting.

By the beginning of this stage the average creatine excretion had risen to 83 mg. (50 to 150) per day. The entire group had lost an average of 100 gm. though five animals still maintained a constant weight. Food intake dropped from an average of 33 gm. to 10 gm. per day and usually reached zero either at the end of this period or early in the next period. The rate of decline in weight was accelerated. During this stage, which lasted 3.5 days (2 to 6), râles became distinctly audible in a majority of the animals.

Stage III. This, the stage of acute dystrophy, lasted 1 to 4 days and terminated in death. The animals were now readily pushed off of their feet, and regained an upright position only after a violent struggle. Some animals died while exhibiting these symptoms, while others were completely prostrated for several days before death. Such animals when picked up seemed to be devoid of all body tonus.

From the beginning of this period until it ended in death, the high creatinuria was maintained, but usually not greatly increased. The daily loss in weight ranged from 50 to 120 gm. The total loss at death averaged 250 gm. Throughout the

course of the disease there was no significant change in the creatinine output.

The most accurate indication of the onset of dystrophy is the increase in creatine excretion. While cessation of growth frequently occurs at about the same time, it can only be distinguished in retrospect from a temporary setback. However, in following the development of the disease, in estimating its severity, and in gauging the proximity of death, all of the described changes are valuable; and they are much more valuable collectively than individually. Nevertheless, special importance is attached to food intake. When an animal's daily food intake has been 10 gm. or less for 4 or 5 days, its condition is critical, and a cure is difficult if not impossible; as will be shown later, however, this is a symptom and not a cause of death.

Autopsies revealed respiratory infections varying in extent from patchy areas on one or two lobes to complete consolidation of most of both lungs. The severity of infection was not correlated with the duration or severity of the symptoms of dystrophy. The one animal free of lung infection was prostrate 2 days before death. With the exception of atrophy of the skeletal muscles, which was frequently apparent, no other macroscopic changes were observed. Microscopic examination of the thigh, diaphragm and masseter muscles revealed necrosis of the muscle fibers as described by Goettsch and Pappenheimer ('31).

The curative factor. Supplements were fed to rabbits with late stage II or stage III dystrophy. In no case was the food consumption allowed to fall below 10 gm. daily for more than 4 days. The creatine excretion exceeded 50 mg. per day. Supplements yielding negative results were refed to animals with early stage I dystrophy. If negative results were again obtained, the ability of the test animal to respond was checked by administering a supplement known to be active. Although a positive response could be detected in 2 or 3 days, the animals were continued for many weeks.

Wheat germ oil. The oil was prepared by extracting fresh wheat germ in a continuous extraction apparatus for 24 hours with cold C.P. anhydrous ethyl ether. Most of the solvent was distilled off at the water pump. The remainder was removed by warming the oil with constant stirring in an open dish on the steam bath.

Levels of 0.5 and 1 cc. per day of this oil were effective in curing dystrophy.

Ferric chloride treated wheat germ oil. Fifty grams of the above oil were dissolved in 250 cc. of ether, shaken with 7 gm. of powdered ferric chloride, and allowed to stand at room temperature overnight. The mixture was then washed repeatedly with water, the ethereal solution dried with sodium sulphate, and the ether removed at the water pump. The resulting dark reddish-brown oil possessed a decidedly acrid odor.

This oil when fed at 3 cc. per day for 7 days gave negative results.

Fractions of the unsaponifiable matter of wheat germ oil. The unsaponifiable matter from 200 gm. of wheat germ oil was partitioned into three fractions by a method previously described by us (Mackenzie et al., '38). These fractions consisted of 6.5 gm. of crystalline ''sterols'' insoluble in methanol at 8°C., 0.8 gm. of an orange wax insoluble in methanol packed in dry ice, and 1.5 gm. of a red-orange oil soluble in methanol packed in dry ice. The latter fraction, in which the vitamin E of the original oil was concentrated, was potent by rat assay in a single 3 mg. dose. Alpha-tocopherol itself is potent at 2 to 3 mg. The wax fraction was subjected to the original procedure and freed from a small amount of the vitamin E concentrate and 0.2 gm. of ''sterols.'' The ''sterol'' fraction was recrystallized from methanol. All fractions were administered in ethyl laurate solutions.

The ''sterol'' and wax fractions were inactive when fed at 32 and 14 mg. per day respectively over a period of 9 days.

The vitamin E concentrate (the oil fraction) was fed at 5 and 25 mg. levels daily to rabbits with stage II dystrophy.

A positive response was detected in 2 days. Cured animals continued on these dosages for 6 to 7 weeks remained in good health and gained from 0.6 to 1 kg. Respiratory infections were absent at autopsy, and microscopic examination of striated muscles revealed no abnormalities.

Ferric chloride treated vitamin E concentrate. The E concentrate was treated in the manner described above for the wheat germ oil. Half a gram of ferric chloride was used for each 100 mg. of the vitamin concentrate. The preparation was inactive when fed at 30 mg. per day for 7 days.

From the foregoing it is apparent that the anti-dystrophy activity in the unsaponifiable matter of wheat germ oil was
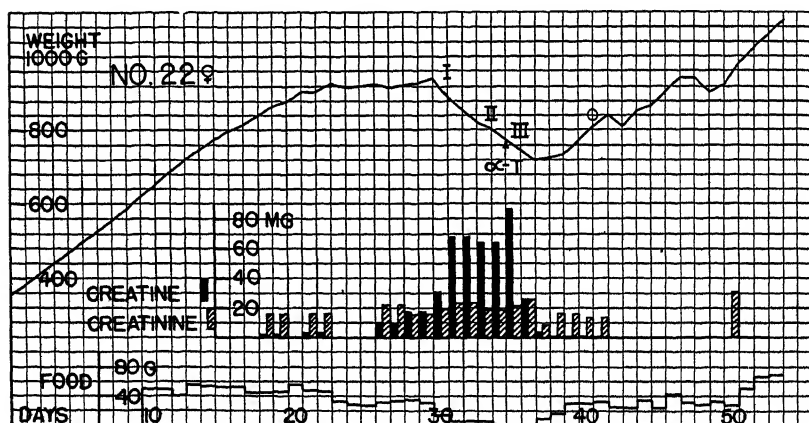
Fig. 1 Rabbit no. 22. Typical cure of dystrophy with alpha-tocopherol. The history of this animal exemplifies those cases in which the disease developed very rapidly (Roman numerals = stages of dystrophy), and in which the rise in urinary creatine was paralleled by a drastic decline in weight and food consumption. By the thirty-fourth day the animal had developed severe râles, and had difficulty in righting when placed on its side. At this time the administration of 5 mg. of alpha-tocopherol daily was commenced (↑). There was a sharp drop in creatine excretion prior to the gain in weight and food consumption. By the seventh day of treatment the animal's movements were normal (O) and the râles had disappeared. For 2 weeks food consumption and growth were below the average for a responding animal, but after that time they reached the average level.

The experiment was continued for 28 weeks, at which time microscopic examination of the biceps femoris revealed only normal muscle fibers.

confined to the fraction which, on the basis of rat assay, consisted of at least 50% vitamin E. Since a positive response could be detected in 3 days (frequently in 2 days) the effective dose of the E concentrate may be calculated as 15 mg. or less, while the ferric chloride treated concentrate was inactive at 210 mg. On the basis of this assumption, ferric chloride destroyed at least 93% of the anti-dystrophy activity.
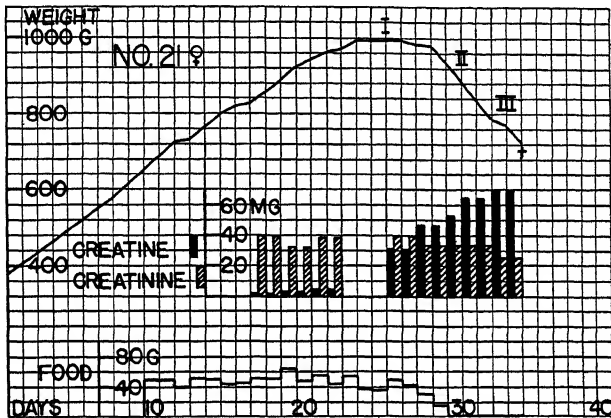
Fig. 2 Rabbit no. 21. Untreated dystrophic animal. This animal was the control of no. 22, figure 1, and like its partner developed acute dystrophy rapidly (Roman numerals = stages of dystrophy). It died (+) with stage III dystrophy on the day alpha-tocopherol therapy was begun on no. 22.

The entire apex of the left lung and part of the apex of the right lung were infected. The biceps femoris showed hyalinized areas in one-third to one-half of the muscle fibers. Many of the hyalin masses appeared to be calcified, while others were undergoing calcification or being absorbed. There was little fibrous tissue and few masses of muscle nuclei. Few polymorphonuclear leucocytes or macrophages were noted.

Similarly, wheat germ oil subjected to the same treatment lost at least 93% of its activity. While these results tend to limit the anti-dystrophy activity to a fraction of the E concentrate sensitive to ferric chloride, the possibility that the neutral fat fraction possesses some activity has not been eliminated.

Alpha-tocopherol.[3] A solution of alpha-tocopherol in ethyl laurate, 20 mg. per cubic centimeter, was employed. Fresh solutions were prepared every 2 weeks and kept in glass-stoppered bottles in the refrigerator. Both natural and synthetic preparations were used. No attempt was made to discern a difference in activity and no difference was observed.

Rabbits with stages II or III dystrophy, weighing approximately 1 kg., were cured by alpha-tocopherol at levels of 3 and 5 mg. per day. Two cured females were maintained in
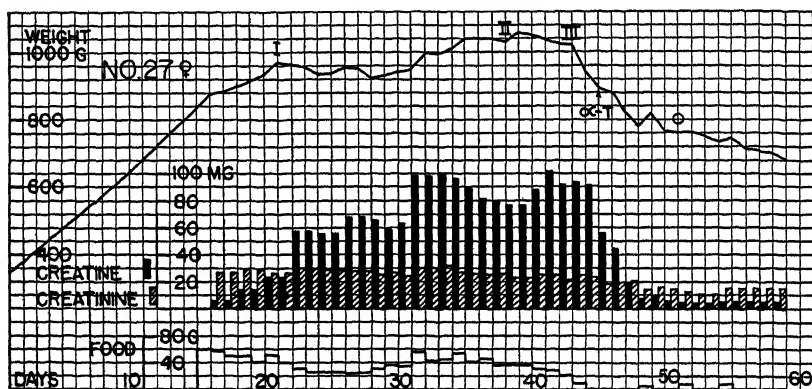
Fig. 3   Rabbit no. 27. Cure of dystrophy with alpha-tocopherol despite continued loss of weight. The history of dystrophy in this animal exemplifies those cases in which the disease developed slowly (Roman numerals = stages of dystrophy) and in which there was a long period of creatinuria during slow growth and fair food consumption. A total of 1.85 gm. of creatine was excreted over a 25-day period.

On the forty-fourth day the animal had acute dystrophy, and when pushed over could only regain its feet after a violent struggle. No rales were observed. At this time treatment with alpha-tocopherol was initiated (↑). Ten milligrams were given on the first 2 days and 5 mg. daily thereafter. The creatine output declined rapidly, but the rabbit refused food and continued to lose weight. A definite improvement in behavior was observed on the fourth day of treatment, and on the sixth day its movements were normal (O).

On the sixtieth day of the experiment (not shown in the figure) a small amount of yeast and an acetone extract of defatted wheat germ were administered and resulted in the immediate resumption of growth and food consumption.

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[3]We are indebted to Merck & Company, Inc., for the supply of alpha-tocopherol.

excellent health on the 3 mg. level for 28 weeks. At the end of that time the animals weighed 2.2 and 3.8 kg. No signs of respiratory infection were found at autopsy, and microscopic examination revealed only normal striated muscle fibers.

In addition to experiments where permanent cures were effected, the duration of a given dose was studied. Animals
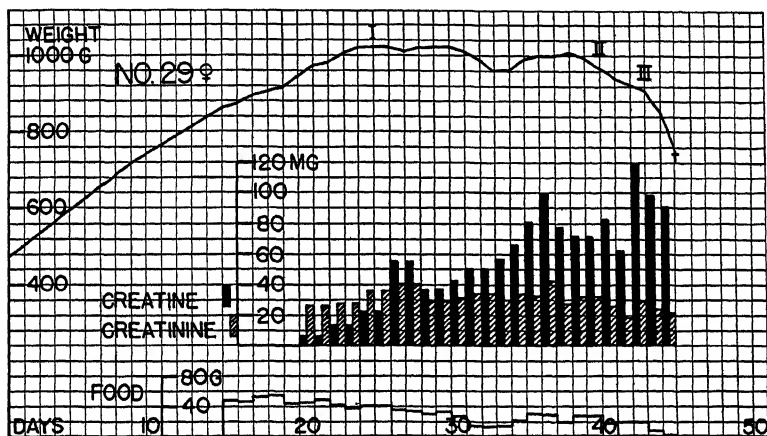
Fig. 4 Rabbit no. 29. Untreated dystrophic animal. This animal was the control of no. 27, figure 3, and like the latter exhibited a prolonged case of the disease (Roman numerals = stages of dystrophy). The animal was prostrate the day before death. Slight râles were noted at the time of death (+), the day on which therapy was started on no. 27.

The respiratory infection was relatively mild, being confined to the apex of one lung. The skeletal muscles were extremely atrophied. Sections of the biceps femoris showed large areas of fibrous tissue and masses of muscle nuclei interspersed with atrophic muscle fibers. Many of the muscle fibers surrounding these areas were hyalinized. There was no indication of calcification as in animal no. 21. A few polymorphonuclear leucocytes and macrophages were present. Similar changes were found in the diaphragm where columns of muscle nuclei were especially prominent.

weighing from 0.7 to 1.2 kg. were cured of stage III dystrophy with 3 mg. of alpha-tocopherol daily for 5 days. The daily creatine excretion fell rapidly to 10 mg. or less. In from 15 to 20 days after the first dose the daily creatine excretion had again risen to 20 mg.

If the rabbit possesses the rat's ability to store vitamin E, the daily requirement of a rabbit on the regimen employed may be put at 0.7 to 1.0 mg. per kilogram of body weight. Additional evidence that the requirement (at least for females) does not exceed this figure was obtained in a prophylactic experiment on two females. These animals, weighing 0.3 and 0.4 kg., were fed 6 mg. of alpha-tocopherol per week for 16 weeks. At the end of this time they were in good health and weighed 2.2 and 2 kg. respectively. Creatine excretion was not studied.

In some cases of very severe dystrophy the feeding of 10 mg. doses of alpha-tocopherol for 2 days, and then dropping to the permanent level, seemed to result in a more dramatic response. However, such a procedure was not essential for curing the disease.

The cure and creatine metabolism. The initial response to vitamin E therapy was a drop in the creatine output from an average level of 80 mg. per day to 46 mg. This occurred within 24 to 48 hours of the feeding of the first supplement. In the latter cases the output for the first 24 hours remained constant or increased. Following this abrupt decline excretion fell to 10 mg. or less in from 1 to 3 days. There was no significant change in creatinine excretion, except in a few cases where there was a relatively small drop of 1 or 2 days' duration, corresponding to the drop in creatine.

One or 2 days after the first decline in the creatine output the animals ceased losing weight, and began to gain from 20 to 100 gm. a day. At the same time the food intake increased, though for a day or 2 it frequently lagged behind the gain in weight by as much as 40 gm.

Definite improvement in the behavior of the animals was noted in from 2 to 4 days after commencing therapy, and in 5 to 7 days their movements were essentially normal. Cured animals resumed a normal rate of growth in 1 to 2 weeks, and gained 150 to 250 gm. a week for 7 to 10 weeks thereafter.

Individual cases selected to illustrate the response to alpha-tocopherol, and the relation of creatine excretion to creatinine excretion, food intake and growth are shown in figures 1, 2, 3 and 4. The selection of female animals for this purpose was fortuitous. Male rabbits behave in a similar manner.

DISCUSSION

The experiments described in this paper show that the muscular dystrophy produced in rabbits reared on an otherwise adequate diet can be permanently cured or prevented by alpha-tocopherol. The requirement of female rabbits for this vitamin, on the diet employed, is not greater than 1.0 mg. per kilo per day. Limited observations on male rabbits indicate a similar requirement. This approximates the fertility requirement of the female rat, if 3 mg. is accepted as the minimum dose for a 200-gm. animal. However, confirmation of the observation of Morgulis et al. ('38) that the lack of a water-soluble factor also produces dystrophy would probably lead to the establishment of a quantitative relationship between the requirements for this factor and alpha-tocopherol. In addition, definite establishment of the requirement depends on the use of a synthetic diet free of rancid fats as well as all traces of vitamin E. Even so, 1 mg. of alpha-tocopherol per kilo per day should prove adequate.

Animals cured of dystrophy that continued to receive alpha-tocopherol showed no return of symptoms even at the end of 28 weeks. The failure of Morgulis et al. ('38) and Morgulis ('38) to effect permanent cures was most likely due to their habit of incorporating the supplements in the diet. A ferric-chloride-treated diet, containing lard and cod liver oil, probably inactivates a considerable portion of any added vitamin E. The inconclusive results obtained by Mattill ('38 b) with vitamin E concentrates may have been due to an involvement of the water-soluble factor.

The experiment of B. H. Thomas et al. ('38, quoted by Mattill, '38 a), indicating the dispensability of vitamin E for reproduction in the rabbit, is difficult to explain. If their diet

was truly E-deficient, then either vitamin E is not the only fat-soluble factor that can prevent dystrophy, or very little is required if a sufficient amount of water-soluble factor is present. Otherwise their animals would all have succumbed of dystrophy long before experiments on reproduction were possible.

As a result of following changes in weight, food consumption, creatine excretion and the alacrity with which the animal moves, we have been able to detect the onset of the deficiency, follow its development and predict with considerable accuracy the time of death. The variations existing between individuals are such that the use of any one of these criteria alone is not satisfactory. Taken alone, a loss of weight would be very misleading in some cases, for several of our animals have lost 100 gm. in 1 week only to gain over 200 gm. during the next 2 weeks. While the creatine output is the most accurate index of the inception of dystrophy (and probably the extent of the lesions) it alone gives no indication of the nearness of death. For this purpose food consumption records and physical symptoms are valuable. Finally, if we were to depend solely on locomotor disturbances we would be led to conclude as was K. Thomas ('38) in a review that, "the dystrophic state sets in very suddenly after a long and uneventful prodromal period."

It is apparent that the absence of vitamin E causes a reduction in food consumption that is responsible for most of the weight loss in dystrophy. Loss of weight, however, does not invariably accompany the development of even severe dystrophy, and hence cannot be considered a cause of the disease.

The enormous increase in creatine excretion does not depend on starvation or loss in weight. This fact is particularly emphasized by those animals that develop a high creatinuria while maintaining a constant weight, and by the sequence of events following vitamin E therapy in which the creatine drops before food consumption is resumed and loss of weight ceases. Neither is the creatinuria due to a disturbance in creatinine elimination, for urinary concentration of the latter

compound remained relatively constant for each animal while that for creatine ranged from 0 to 150 mg. It may be concluded, therefore, that the creatinuria is a result of some direct effect of the vitamin deficiency on the organism. In view of this and Goettsch and Brown's ('32) observation that the creatine content of striated muscle is greatly reduced in dystrophic rabbits, it is extremely probable that the urinary creatine arises from muscle creatine.

While it seems probable that this loss of muscle creatine is a consequence of the muscle degeneration, we have no evidence that a disturbance in creatine or phosphocreatine metabolism is not the primary change in the muscle. In either case the response to vitamin E given by mouth as gauged by the drop in creatine excretion is extremely rapid, 1 or 2 days, suggesting that the vitamin acts directly on the muscle. In this connection it is interesting to recall the early observation of Evans ('32) that muscle is one of the richest animal sources of vitamin E. Verzár's ('39) recent observation that adult female rats developing paralysis and muscular dystrophy on a vitamin E-deficient diet exhibit a creatinuria which requires 200 mg. of alpha-tocopherol daily for its complete suppression is difficult to explain at the present time.

The rabbit offers excellent possibilities as an animal for vitamin E assay provided that a constant ratio exists between anti-sterility and anti-dystrophy activity. That this may not be the case is indicated by the work of Goettsch and Ritzmann ('39). Such a situation would necessitate specifying whether anti-sterility or anti-dystrophy activity is referred to when using the term "vitamin E potency." Dystrophy develops more rapidly in young rabbits than sterility in female rats, and by using creatine excretion as the basis for a test the response to a positive supplement can be detected in several days. While it may prove difficult to place this procedure on a strictly quantitative basis, a very rapid semi-quantitative method is certainly feasible.

Until very recently no species were known with certainty to require vitamin E other than the rat and mouse, and in

these two species its absence did not appear to exert a very profound effect on the well-being of the adult. Now it is known to be essential for life in the rabbit (Mackenzie and McCollum, '39), guinea pig (Shimotori et al., '39) and chicken (Dam et al., '38); and, in view of these developments, probably for life in the duck (Pappenheimer and Goettsch, '34) and goat (Madsen et al., '33). It is indispensable for the prevention of paralysis and muscular dystrophy in the suckling (Barrie, '38; Goettsch and Ritzmann, '39) and adult rat (Mackenzie et al., '39; Verzár, '39). Its elevation from a relatively obscure position among the vitamins to one of prominence would seem to warrant a reexamination of its distribution in nature.

SUMMARY

1. Nutritional muscular dystrophy in the rabbit resulting from a deficiency of a fat-soluble factor is cured by alpha-tocopherol. Continued administration of the vitamin prevents a recurrence of the disease even after acute cases. The anti-dystrophy requirement of the rabbit for alpha-tocopherol does not exceed 1.0 mg. per kilo per day.

2. The activity of the unsaponifiable matter of wheat germ oil is confined to a fraction very rich in vitamin E. The anti-dystrophy factor in this concentrate is destroyed by ferric chloride treatment.

3. Simple criteria have been established for following the development of dystrophy, and for detecting a response to potent supplements within several days.

4. A great increase in urinary creatine invariably attends the deficiency and may precede the gross symptoms by 2 weeks or more. The excretion of creatine is not due to loss of weight or starvation. A marked reduction in urinary creatine occurs within 24 or 48 hours of vitamin E administration. No comparable changes occur in creatinine metabolism.

5. Consideration of the rabbit as a test animal for the biological estimation of vitamin E is suggested.

The histological sections used in this experiment were pre-
pared by Miss Miriam Reed.

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THE AMINO ACIDS REQUIRED FOR THE COMPLETE REPLACEMENT OF ENDOGENOUS LOSSES IN THE ADULT RAT [1]

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If the endogenous catabolism of nitrogen can be neither depressed nor stimulated by the feeding of amino acids or proteins, as our experimental results reported elsewhere ('40) indicate, then the changes in the output of urinary nitrogen induced by the feeding of amino acids may be given their obvious interpretation with reference to the utilization of dietary amino acids. Also, the accompanying change in the balance of nitrogen may be interpreted as a measure of the utilization of dietary nitrogen in anabolism rather than as a resultant of an indeterminate depression of endogenous losses of nitrogen associated with an incommensurable utilization of the dietary amino acids themselves. In particular, the attainment of nitrogen equilibrium by feeding an appropriate amino acid mixture to an animal may be clearly interpreted as a complete replacement of the losses of nitrogen resulting from a constant attrition of nitrogenous tissue components, not as a complete suppression of such attrition. The experiments to be reported in this paper, concerned with the determination of the amino acids required preformed in the diet for the attainment of nitrogen equilibrium, will be interpreted in this simple and direct manner.

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[1] The substance of this paper was taken from a thesis submitted by E. Wise Burroughs to the Graduate School of the University of Illinois in partial fulfillment of the requirements of the degree of doctor of philosophy in animal husbandry, July, 1939.

The early investigations on this subject are reviewed in previous publications from this laboratory (Mitchell, '16; Geiling, '17). Recently Wolf and Corley ('39) have reported the results of experiments on adult rats concerned with the amino acids required for nitrogen equilibrium. They conclude that all of the amino acids except arginine that Rose ('38) has found to be essential for growth, are also essential for maintenance in the above sense, "as omission of each of these amino acids from the complete control diet has been followed by a negative balance of nitrogen, while subsequent replacement has been followed by a restoration of nitrogen equilibrium." Unfortunately there is evidence in the representative data selected for presentation that the 3-day periods into which the experiments were divided were not sufficiently long for the rats to adjust themselves to the dietary changes made. Consequently, the evidence for the indispensability of some of the amino acids, particularly lysine and histidine, is not conclusive. Also, the possibility of the conversion of a dispensable amino acid into an indispensable one at a rate adequate for the maintenance of nitrogen equilibrium if not for growth, is not even tested. Whether tyrosine can replace phenylalanine in adult nutrition, for example, cannot be answered by the experiments of Wolf and Corley.

A note by Rose and Rice ('39) concerning the significance of the amino acids in adult canine nutrition states that "the qualitative amino acid needs of the dog are identical with those of the rat" on the basis of nitrogen metabolism studies on dogs conducted apparently according to much the same plan as the experiments of Wolf and Corley, though the periods were of 7 days' duration. Until the details of these experiments become available, critical evaluation of them is obviously impossible.

There are indications, however, that the amino acid requirements of maintenance are in some particulars qualitatively different from those of growth. This evidence is very suggestive with reference to lysine. The early experiments of Osborne and Mendel ('16) showed clearly that tryptophane

was essential for both maintenance and growth, but that zein, which contains no lysine (Vickery, '38), when supplemented with tryptophane, will maintain rats at constant body weight for as long as 6 months. These experiments fall somewhat short of demonstration of the dispensability of lysine for maintenance by reason of the fact that the rations contained 28% of "protein-free milk," containing 0.7% of nitrogen (Osborne, Mendel and Ferry, '12). However, even assuming that all of this nitrogen is in the form of protein, the daily intake of protein corresponding to a food intake of 5 gm. would be no greater in amount than that contained in 200 mg. of yeast, an addendum quite commonly used in later amino acid studies in which "protein-free milk" has been dispensed with. Geiling's work ('17) also supports the view that lysine may be dispensable for maintenance.

Later experiments on the availability of lysine and its derivatives for growth have involved the feeding of zein diets to which small addenda of tryptophane and of yeast vitamin concentrate [2] have been added. In these experiments, on the basal unsupplemented diet, continued maintenance or even slow growth was observed (Berg, '36; Conrad and Berg, '37 a; Totter and Berg, '39) with rats or mice. The conclusion of Morris and Wright ('35) that there is a definite lysine requirement for the maintenance of cattle is based upon the wholly gratuitous assumption that the low biological value of wheat gluten for maintenance is the result of its low content of lysine. Osborne and Mendel ('19) have shown that the proteins of the wheat endosperm are practically as valuable for the maintenance of rats as the proteins of the whole wheat kernel, although for growth they are markedly inferior.

Many experiments on the availability of histidine and histidine derivatives for growth have shown that on the histidine-low basal diets used, containing casein hydrolysates from which the histidine was removed by mercuric sulfate or by electrodialysis, plus the usual addenda of yeast or

[2] From the Harris Laboratories, Tuckahoe, N. Y.

yeast concentrate, rats may maintain body weight for periods ranging from 20 days to 100 days (Cox and Berg, '34; Fishman and White, '36; du Vigneaud, Sifferd and Irving, '37; Conrad and Berg, '37 b). These experiments gain added significance from the fact that yeast contains only about 1% of histidine (Woolley and Peterson, '37).

Such evidence as that cited points to the possibility that lysine and histidine may not be required for the maintenance of nitrogen equilibrium in adult rats.

EXPERIMENTAL METHODS

The method used in determining the amino acids required to replace the endogenous losses of nitrogen in adult rats was similar to that used by Wolf and Corley, namely, the determination of the effect on the nitrogen balance of the successive withdrawal of individual amino acids, attention being confined to the ten amino acids considered by Rose to be indispensable for growth. However, certain important differences in procedure should be noted:

1. The complete amino-acid mixture used contained not only the ten indispensable acids, but also as many of the dispensable ones as were available. Thus, the possibility of the conversion of a dispensable acid into an indispensable one at a rate sufficiently rapid for the maintenance of nitrogen equilibrium, though not rapid enough for appreciable growth, could be explored.

2. No supplements of the B-complex vitamins were fed during the experimental periods with the exception of a daily dose of 0.1 mg. of crystalline thiamin hydrochloride. The necessity of adding other nitrogen-containing vitamin supplements was obviated by incorporating yeast concentrate [3] in the diet of the preliminary feeding period of 12 to 14 days. Advantage was thus taken of the known capacity of the animal body to store the various factors in the vitamin B_2 complex, especially in the liver. It was hoped that this storage would

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[3] See footnote 2, p. 365.

not be depleted during the period of experimental feeding, but if depletion did occur there seems to be sufficient evidence in the literature (Karr, '20; Fixsen, '30; Kon, '31) to the effect that the metabolism of nitrogen would not be adversely affected provided the intake of food was sufficient to cover the energy requirements.

3. Throughout the experimental feeding periods with each rat, the same amount of food was given. Early in the investigation it was found that not only were the incomplete diets unpalatable, but the same was also true of the complete mixtures. The rats would voluntarily eat a fair portion of their food, but not in amounts sufficient to maintain body weight, nor would they eat even approximately the same amount each day. Since the consumption of an adequate and a constant amount of food is of paramount importance in a nitrogen balance study, each rat was force-fed his entire ration twice daily by a method patterned after the technic that Wolfe ('38) used in administering amino acid mixtures to rats.

Adult female albino rats, weighing approximately 200 gm. (170 to 250 gm.), served as subjects of the experiment. They were first fed a standardizing diet for a period of at least 12 to 14 days. This ration contained only about 5% of protein and was well supplied with the B vitamins. The purpose of this period was not only to replenish the stores of the B_2 vitamins, but also to deplete the stores of ''deposit protein'' and to reduce all rats to a uniform low level of protein nutrition.

Following the standardizing period the rats were placed upon a nitrogen-free diet containing, in per cent: starch 63, sucrose 10, salt mixture (modified Osborne and Mendel) 5, lard 10, butterfat 10, cod liver oil 1.5, and wheat germ oil 0.5. In addition, 1 or 2 gm. of sucrose were given to each rat daily, the amount depending on the ability of the rat to maintain body weight. On the second day on this diet, each rat was given a complete amino-acid mixture equivalent to 80 mg. of nitrogen daily, and the supplemented ration was

fed until daily positive and approximately uniform nitrogen balances were obtained, ordinarily requiring from 5 to 10 days. The level of amino-acid feeding was selected on the basis of preliminary experiments as being adequate for nitrogen equilibrium but not excessive. The nitrogen in the complete amino acid mixture was divided equally among the following twenty amino acids: *dl*-threonine, *dl*-isoleucine, *l*-tryptophane, *dl*-valine, *dl*-methionine, *dl*-lysine, *l*-histidine, *dl*-phenylalanine, *dl*-leucine, *d*-arginine, *l*-cystine, *l*-tyrosine, glycine, *dl*-alanine, *dl*-serine, *dl*-norleucine, *dl*-aspartic acid, *dl*-glutamic acid, *l*-proline, and *l*-hydroxyproline.

In the next experimental period amino acids were withdrawn individually from the mixture, to determine the effect on the nitrogen balance. The incomplete mixtures were also fed at a rate equivalent to 80 mg. of nitrogen per rat per day. In this manner the dispensability for nitrogen equilibrium of the ten amino acids considered indispensable for growth by Rose was tested, and also the dispensability of cystine and tyrosine. These deficient mixtures were fed until uniform daily nitrogen balances were obtained, requiring usually from 3 to 5 days, though in a few cases the period was extended to 8 days.

The rats were then returned to the complete mixture of amino acids used in period 1. This period was continued until consistent positive nitrogen balances were secured, or until it was evident that positive balances could not be obtained. Only in the former case was the test considered successful.

Throughout these three periods, each rat received the same amount of food daily, generally 8 gm., and the same amount of nitrogen, 80 to 84 mg., of which 3 to 4 mg. were supplied by the basal diet. The metabolism cages and the methods of collection and analysis of excreta are described elsewhere (Burroughs, Burroughs and Mitchell, '40). However, in the experiments to be described below, analyses were made on the daily samples of urine. The feces were composited and analyzed for the various experimental periods.

The proof of the purity of the amino acids used in these experiments rests upon the analyses for total nitrogen, or for amino nitrogen by the Van Slyke method. The agreement with the theoretical percentages was within 0.2%, with the exception of *l*-proline (0.25%) and of *d*-arginine monohydrochloride. In the latter case the discrepancy was found to be due to a contamination with mineral matter amounting to 5%. In order best to avoid contamination of one amino acid with another, synthetic racemic mixtures were used whenever obtainable.

DISCUSSION OF EXPERIMENTAL RESULTS

Representative results obtained with the twelve amino acids that were studied by the method of successive withdrawal from the complete amino-acid mixture are presented in the chart. With reference to these results, the following points of interest may be mentioned.

The withdrawal of only five amino acids produced a marked negative nitrogen balance in the experimental rats. Somewhat in the order of the magnitude of the negative balance produced, the amino acids are: threonine, isoleucine, valine, tryptophane and methionine. With the first three of these acids, the change from a positive to a negative nitrogen balance was abrupt; with tryptophane and methionine a lag of 1 to 3 days occurred after the withdrawal of the acid before the loss of nitrogen from the body exceeded the intake. Withdrawal of methionine from the ration produced a progressive increase in negative nitrogen balance throughout the period during which such balances were obtained. There is no question but that these five amino acids are essential dietary constituents for the maintenance of nitrogen equilibrium in the adult rat.

The relationship between methionine and cystine revealed by these studies is shown by the data summarized in table 1. The delayed appearance of the disturbance in nitrogen metabolism occasioned by the withdrawal of methionine from the diet is shown best in the case of rat 24, which maintained

a distinctly positive balance for 4 days on a methionine-free diet. The data on rats 24 and 51 show that a methionine deficiency cannot be compensated for by the addition of cystine. On the other hand, a negative balance of nitrogen brought about by the withdrawal of cystine from the diet (rat 36) can be corrected by an addition of methionine. In the case of rat 38, the same effect was shown, but the period

AMINO ACID REQUIREMENTS FOR MAINTENANCE AS MEASURED BY THE NITROGEN BALANCE METHOD.
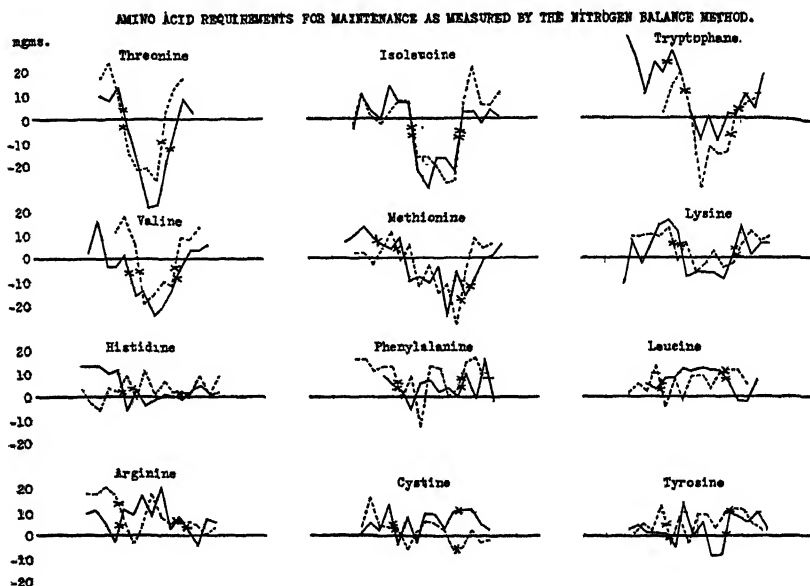
Fig. 1 Each pair of lines represents nitrogen balances with two female rats. Points between x——x represent days on respective deficient amino acid mixtures. Points preceding or following x——x represent days on complete amino acid mixture.

was not continued long enough, apparently, for equilibrium to be established. The experiments indicate clearly that there is a body requirement for both cystine and methionine, but that dietary cystine can cover only the cystine requirement, while dietary methionine can cover the requirements for cystine as well as for methionine.

The evidence for the dispensability for the maintenance of nitrogen equilibrium of some of the amino acids that are

indispensable for growth requires more complete presentation than is afforded by the chart. Hence, in tables 2 to 5, inclusive, the period averages for individual rats have been tabulated, embodying all of the evidence obtained with reference to

TABLE 1

The significance of methionine and cystine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM THE COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
20	6	None	198	0	82.7	69.4	8.4	+4.9
	4	Methionine		—3	83.0	78.6	11.8	—7.4
	8	None		—3	82.7	72.3	7.4	+3.0
24	3	None	190	+2	81.2	62.9	8.1	+10.2
	4	Methionine		+1	82.4	66.9	8.6	+6.9
	3	Methionine		+3	82.4	83.6	8.6	—9.8
	4	Methionine[1]		+3	82.4	86.7	8.6	—12.9
	4	None		0	81.2	67.2	12.2	+1.8
51	5	None	215	+11	83.8	69.5	11.2	+3.1
	2	Methionine [1]		+1	83.4	66.4	14.5	+2.4
	5	Methionine [1]		—4	83.4	83.1	14.5	—14.2
	4	None		—4	83.8	68.7	8.9	+6.2
36	4	None	210	+3	81.2	76.1	7.2	—2.1
	4	Cystine		—10	82.3	97.1	9.2	—24.0
	1	Cystine [2]		—2	82.3	94.9	9.9	—22.5
	2	Cystine [2]		—1	82.3	65.4	9.9	+7.0
	4	None		0	81.2	72.4	8.3	+0.5
38	4	None	231	+1	81.2	68.7	11.6	+0.9
	4	Cystine		—8	82.3	96.4	12.5	—26.6
	3	Cystine [2]		—4	82.3	76.3	12.8	—6.8
	4	None		+4	81.2	69.6	9.4	+2.2

[1] In these periods the proportion of cystine in the amino acid mixture was doubled.

[2] In these periods the proportion of methionine in the amino acid mixture was doubled.

these amino acids except the few unsatisfactory experiments in which a positive nitrogen balance could not be established in the final period on the complete amino-acid mixture.

The withdrawal of lysine from the diet (table 2) was associated with only small losses of nitrogen daily, and when

the norleucine content of the ration was doubled, the average loss of nitrogen over a 7-day period was only 1.5 mg. daily. For the individual days of this period the balances ranged from —7.2 to +6.4 mg. of nitrogen. For rats 21, 22 and 50, the average daily excretion of urinary nitrogen definitely increased in the second experimental period when lysine was withdrawn from the diet, but in the case of rat 75, for which the nitrogen intake was reduced to 33.5 mg. daily, less than the endogenous loss of nitrogen for a rat of this size, the

TABLE 2

The significance of lysine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
21	6	None	196	0	82.7	66.6	10.4	+5.7
	7	Lysine		—5	82.1	75.0	11.2	—4.1
	4	None		—4	82.7	68.1	9.6	+5.0
22 ·	4	None	193	+2	82.7	63.2	8.0	+11.5
	5	Lysine		—2	82.1	82.3	7.4	—7.6
	5	None		—2	82.7	68.2	9.2	+5.3
50	5	None	225	+4	83.8	67.3	6.8	+9.7
	7	Lysine [1]		—3	82.6	73.2	10.9	—1.5
	4	None		—2	83.8	67.2	8.6	+8.0
75	4	None	183	—4	33.5	41.3	10.2	—18.0
	5	Lysine [1]		—3	33.5	42.9	10.2	—19.6
	4	None		—2	33.5	42.6	9.3	—18.4

[1] During this period, the proportion of norleucine in the amino acid mixture was doubled.

urinary nitrogen was not appreciably increased by the withdrawal of dietary lysine. The evidence suggests rather strongly, but unfortunately does not prove, that lysine is a dispensable dietary component in the maintenance of the nitrogenous integrity of the tissues.

As regards phenylalanine and tyrosine, the data in table 3 show clearly (1) that a diet lacking either of these amino acids but containing proper amounts of the other is compatible with nitrogen equilibrium, (2) that the withdrawal of

TABLE 3

The significance of phenylalanine and tyrosine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
30	3	None	197	+1	81.2	57.1	7.7	+16.4
	8	Phenylalanine		+1	80.4	78.0	6.9	—4.5
	3	None		—4	81.2	70.5	6.9	+3.8
25	2	None	196	—5	81.2	68.0	6.5	+6.7
	7	Phenylalanine [1]		—3	80.4	72.5	6.0	+1.9
	4	None		—3	81.2	66.9	8.3	+6.0
53	5	None	207	+3	83.3	62.8	6.8	+13.7
	7	Phenylalanine [1]		0	82.1	68.4	10.6	+3.1
	4	None		—1	83.3	63.0	8.4	+11.9
76	3	None	184	—1	33.5	39.0	11.6	—17.1
	5	Phenylalanine		—4	33.5	40.3	8.4	—15.2
	4	None		—2	33.5	44.5	12.2	—23.2
31	5	None	185	—6	81.2	71.1	8.1	+2.0
	6	Tyrosine		—3	83.1	76.0	7.9	—0.8
	5	None		—4	81.2	65.8	7.9	+7.5
32	5	None	195	0	81.2	72.4	8.7	+0.1
	6	Tyrosine		—4	83.1	76.4	9.5	—2.8
	5	None		0	81.2	72.8	9.5	—1.1
62	4	None	236	+11	83.8	68.2	10.0	+5.6
	4	Tyrosine, phenylalanine		—3	83.3	78.2	9.7	—4.6
	4	Tyrosine, phenylalanine		—5	93.2	82.4	17.7	—6.9
63	4	None	242	+6	83.8	67.9	7.0	+8.9
	4	Tyrosine, phenylalanine		0	83.3	77.6	9.4	—3.7
	4	Tyrosine, phenylalanine		+2	93.2	99.0	7.4	—13.2
	4	Tyrosine [2]		—2	94.2	70.4	11.4	+12.4
	4	Phenylalanine [1]		0	92.4	79.6	11.4	+1.4

[1] During these periods the proportion of tyrosine in the amino-acid mixture was doubled.

[2] In this period the proportion of phenylalanine in the amino-acid mixture was doubled.

phenylalanine from an amino-acid mixture, fed at a low level of intake and containing a proper amount of tyrosine, does not induce an increase in the nitrogen output in the urine (rat 76), but (3) that the withdrawal of both tyrosine and phenylalanine from the diet simultaneously is incompatible with nitrogen equilibrium. The evidence quite clearly indicates that phenylalanine and tyrosine are interchangeable in adult nutrition.

TABLE 4

The significance of leucine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
23	4	None	193	+3	81.2	56.7	8.8	+15.7
	6	Leucine		−1	82.5	55.7	6.7	+20.1
	5	None		−2	81.2	61.0	5.4	+14.8
24	2	None	191	−3	81.2	68.7	8.2	+4.3
	7	Leucine		+1	82.5	64.9	6.5	+11.1
	4	None		−2	81.2	71.1	7.9	+2.2
48	4	None	210	−1	83.0	69.6	8.0	+5.4
	7	Leucine, tyrosine, cystine [1]		−2	82.1	65.4	12.1	+4.6
	4	None		−6	83.0	66.1	9.6	+7.3
49	4	None	203	+1	83.0	67.8	8.5	+6.7
	7	Leucine, tyrosine, cystine [1]		−4	82.1	72.6	9.1	+0.4
	4	None		−3	83.0	74.3	10.1	−1.4
70	3	None	182	−7	82.2	64.8	8.5	+8.9
	4	Leucine, norleucine		−9	81.9	81.1	8.5	−7.7
	3	None		+6	82.2	65.4	8.2	+8.6

[1] During these periods the proportions of both phenylalanine and methionine in the amino-acid mixture were doubled.

The feeding of a leucine-free amino-acid mixture to rats 23 and 24 (table 4) neither increased the nitrogen output in the urine nor induced a negative balance of nitrogen during periods of 6 or 7 days duration. To meet the objection that leucine may be present in some of the naturally-occurring amino acids as a contamination without modifying materially

their observed contents of nitrogen, two rats, nos. 48 and 49, were fed a diet lacking leucine and also tyrosine and cystine, the amino acids most likely to be contaminated with leucine. Again, positive nitrogen balances were obtained throughout 7-day periods. It may be concluded, therefore, that leucine is dispensable from the diet for the mere maintenance of nitrogen equilibrium. The results on rat 70, indicating a negative nitrogen balance when both leucine and norleucine are withdrawn from the diet simultaneously point toward the conclusion, indefensible from the biochemical standpoint, that norleucine may be convertible into leucine in the body, on the assumption that norleucine itself is a non-essential dietary component.

The metabolism data summarized in table 5 demonstrate that neither histidine nor arginine are essential in the maintenance of nitrogen equilibrium. In order to insure the validity of this conclusion, the histidine-free amino acid mixtures fed to rats 52 and 64, as well as all naturally-occurring amino acids in them, were examined for the presence of histidine by means of the Knoop bromine test. The test was negative in all cases. Also, the withdrawal of both histidine and arginine failed to induce a negative nitrogen balance in rat 69.

The above experiments indicate, with more or less certainty, that the adult rat needs only the following amino acids in the function of replacing completely the loss of nitrogen in the endogenous catabolism: threonine, isoleucine, tryptophane, valine, methionine, phenylalanine or tyrosine, and leucine or norleucine. The evidence with reference to the replaceability of leucine by norleucine is admittedly weak, and that for the dispensability of lysine is not particularly strong, and here also the function of norleucine as a precursor or substitute may enter in. A crucial test of this proposition would be one concerned with the adequacy of such an amino acid mixture in replacing the endogenous losses of nitrogen in adult rats. A test on three female rats was therefore undertaken with the following mixture, the percentages indicating the distribution of nitrogen: threonine 15, isoleucine 15,

tryptophane 6, valine 12, methionine 12, norleucine 30, and tyrosine 10. The proportions of the amino acids in this mixture were quite arbitrarily selected. The rats, previous to the test, had been subsisting on the basal nitrogen-free diet.

The data obtained with one of the rats, no. 71, during an 8-day feeding period, were quite satisfactory in establishing

TABLE 5

The significance of histidine and arginine in the endogenous metabolism

RAT NO.	LENGTH OF PERIOD	AMINO ACIDS OMITTED FROM COMPLETE MIXTURE	INITIAL BODY WEIGHT	GAIN OR LOSS IN WEIGHT	AVERAGE VALUES PER DAY:			
					Nitrogen intake	Urinary nitrogen	Fecal nitrogen	Nitrogen balance
	days		*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
13	3	None	218	+1	82.5	64.5	10.2	+7.8
	9	Histidine		−6	82.5	54.2	10.1	+18.2
	3	None		−3	82.5	60.7	6.5	+15.3
52	5	None	215	−1	83.3	63.6	7.7	+12.0
	6	Histidine [1]		−5	82.4	74.4	9.1	−1.1
	4	None		−2	83.3	72.0	9.0	+2.3
64	3	None	251	−2	93.7	77.1	11.6	+5.0
	6	Histidine [1]		+1	92.7	77.5	11.6	+3.6
	4	None		−6	93.7	78.2	10.1	+5.4
35	4	None	210	+1	81.2	67.4	8.6	+5.2
	6	Arginine		0	81.5	63.0	6.8	+11.7
	5	None		−2	81.2	71.3	6.6	+3.3
39	4	None	207	+1	83.0	54.2	10.1	+18.7
	7	Arginine [2]		−5	81.6	64.6	10.9	+6.1
	4	None		−1	83.0	66.8	13.2	+3.0
69	3	None	172	−1	82.2	61.0	7.7	+13.5
	5	Arginine, histidine		−3	82.6	63.5	6.8	+12.3
	3	None		0	82.2	63.1	6.3	+12.8

[1] In these periods the proportion of arginine in the amino-acid mixture was doubled.

[2] In this period the proportion of histidine in the amino-acid mixture was doubled.

the adequacy of the amino-acid mixture (table 6). Positive nitrogen balances were obtained on all but 2 of the 8 experimental days, and the average balance for the period was +2.1 mg. of nitrogen per day. The body weight was also maintained after a slight initial loss. The other two rats,

on the same amino-acid mixture (containing a new supply of four of the amino acids) lost weight rapidly (3 to 5 gm. daily), exhibited a pronounced negative nitrogen balance and seemed to be in constant agony. That these events were not the result of an amino-acid deficiency is shown by the fact that they were not alleviated by the addition of histidine,

TABLE 6

The nitrogen metabolism of rats on a simplified amino-acid mixture [1]

DAY	BODY WEIGHT	FOOD INTAKE	DAILY NITROGEN METABOLISM:			
			Intake	Urine	Feces	Balance
	gm.	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
			Rat 71			
1	175	7.0	81.3	71.5	8.2	+1.6
2	174	7.0	81.3	68.0	8.2	+5.1
3	175	7.0	81.3	71.5	8.2	+1.6
4	172	7.0	81.3	76.0	8.2	—2.9
5	172	7.0	81.3	65.9	8.2	+7.2
6	172	7.0	81.3	73.7	8.2	—0.6
7	172	7.0	81.3	71.0	8.2	+2.1
8	172	7.0	81.3	70.2	8.2	+2.9
Average	173	7.0	81.3	71.0	8.2	+2.1
			Rat 77			
1	188	7.0	80.9	51.2	6.8	+22.9
2	188	7.0	80.9	79.9	6.8	—5.8
3	189	7.0	80.9	72.4	6.8	+1.7
4	188	7.0	80.9	104.9	6.8	—30.8
5	186	7.0	80.9	70.7	6.8	+3.4
6	188	7.0	80.9	72.4	6.8	+1.7
Average	188	7.0	80.9	75.2	6.8	—1.1
			Rat 78			
1	193	7.0	80.9	67.1	5.7	+8.1
2	194	7.0	80.9	79.1	5.7	—3.9
3	194	7.0	80.9	76.3	5.7	—1.1
4	194	7.0	80.9	82.4	5.7	— 7.2
5	192	7.0	80.9	77.0	5.7	—1.8
6	194	7.0	80.9	75.5	5.7	—0.3
Average	194	7.0	80.9	76.2	5.7	—1.0

[1] The distribution of nitrogen in the amino acid mixture given to rat 71 was as follows in per cent: threonine 15, isoleucine 15, tryptophane 6, valine 12, methionine 12, norleucine 30, and tyrosine 10.

The mixture fed to rats 77 and 78 was the same as the above mixture with the exception of the substitution of leucine for norleucine.

lysine, leucine and phenylalanine to the diet. They suggest a toxicity of some constituent of the diet, and on canvassing the situation it appeared that the most likely culprit was the norleucine, since Womack and Rose ('36) had reported somewhat similar (though less severe) experiences with this amino acid (see also Rose, '38).

Therefore, the amino-acid mixture was changed by the substitution of leucine for norleucine, and the experiment was repeated on two other rats, nos. 77 and 78, with the results assembled in table 6. The irregular nitrogen balances of rat 77 on the first and the fourth day of the experiment are quite unusual and inexplicable. For both rats the average daily nitrogen balance for the 6-day period was very close to zero, i.e., —1.2 and —1.0 mg., indicating essential equilibrium, perhaps within the limits of error of the methods used. The authors believed that in all probability positive balances would have been obtained if the intake of the simplified amino-acid mixture had been raised. In any case, the positive results obtained with rat 71 are sufficiently clear-cut to establish the adequacy of the amino acids fed.

The fact that the rat needs ten amino acids in the diet for growth and only seven for the replacement of endogenous losses can be explained on the same general basis as that used by Rose (Westerman and Rose, '28) in explaining the inability of α-di-hydroxy-β-dithiodipropionic acid to replace cystine in the diet, and later (Rose, '38) in the evaluation of arginine for growth. In the former case, the negative experimental results obtained were explained as follows: "Either the amino acid cannot be formed from the corresponding hydroxy acid under the conditions of our experiments, or its synthesis is not sufficiently rapid to meet the growth requirements of the organism." In the latter case, in view of the fact that the body's ability to synthesize arginine had been established (Scull and Rose, '30), but that in the absence of arginine from the diet rats "invariably gain only about 70 to 80% as much as controls which receive this amino acid" (Rose, '38), arginine is classed as an indispensable amino

acid in the sense that it "cannot be synthesized by the animal organism, out of the materials ordinarily available (Cox and Rose, '26) at a speed commensurate with the demands for normal growth." In the same vein, he predicts: "For adult animals it may prove to be dispensable inasmuch as its synthesis may proceed at a rate equal to the requirements of maintenance alone." His own results, but recently announced (Rose and Rice, '39) confirm this prediction, as do those reported in this paper.

The hypothesis that certain of the body's synthetic capacities set the pace for growth, analogous to Robertson's ('23) "master reactions," and may even limit growth to a rate that is subnormal compared to what may be attained under adequate dietary conditions, receives support from evidence outside the field of protein metabolism. The tissues of the guinea pig apparently can synthesize ascorbic acid (Wachholder, Baucke and Podesta, '39), but certainly not at a rate to permit normal growth and health. On the other hand, the mouse, in spite of its ability to synthesize ascorbic acid (Beard, '25–'26; Harde and Wolff, '34) may not be able to grow and maintain health on diets devoid of the vitamin (Kleiner and Tauber, '36). Such evidence suggests the possibility that specific differences with respect to vitamin requirements may not be as sharp as is commonly supposed, but may be an expression merely of differential rates of synthetic reactions.

In lactation, an analogous situation exists with reference to the synthesis of fat. The ability of the body to synthesize fat from carbohydrate was one of the first synthetic reactions demonstrated in animal nutrition. However, a modicum of fat in the diet is essential for the maximum performance of this function (Maynard and McCay, '32; Mackenzie, Mackenzie and McCollum, '39).

Thus, the results of this investigation, insofar as they differ from the growth investigations of Rose, may be explained on the basis of the relation between the rate of synthesis of an amino acid and the physiological demand for it in growth

as compared to the reconstruction of tissue constituents containing it necessitated by their endogenous catabolism.

Lysine may be synthesized at a rate compatible with the replacement of endogenous losses of it, but incompatible with the demands of growth or of reproduction in the female (Pearson, '37; Lafon and Veillet, '38). The same situation may exist with reference to histidine and leucine. However, the alternative interpretation that the body does not need these amino acids in the replacement of endogenous losses is seemingly as valid as the one just given, except possibly in the case of histidine. Besides its occurrence in body proteins, histidine occurs in, or is related to, such compounds as ergothionine, carnosine and histamine that occur in the tissues and would presumably be involved in the endogenous catabolism.

The apparent interchangeability of phenylalanine and tyrosine may be at such a slow rate as to be without significance in growth, although adequate for the demands for nitrogen equilibrium. The conversion of phenylalanine into tyrosine in the body is strongly suggested by the experimental findings of Embden and Baldes ('13) and of Kotake, Masai and Mori ('22), without, however, any implication that this is necessarily the normal pathway for the metabolism of phenylalanine. However, no evidence for the conversion of tyrosine to phenylalanine has ever been obtained and the fact that the reduction of phenolic substances such as tyrosine to unsubstituted benzene derivatives has, according to Dakin ('22), not yet been observed in the animal organism, suggests "that the nuclear oxidation of phenylalanine is an irreversible reaction." The facts that phenylalanine but not tyrosine is required for growth (Womack and Rose, '34), that either one may serve for the maintenance of nitrogen equilibrium (this investigation), and that phenylalanine may be converted to tyrosine in the body while the inverse is improbable, can best be reconciled on the assumption that the body needs both amino acids for the synthesis of protein in growth, but needs only tyrosine in the replacement of endogenous

losses. The requirements of growth can be covered by phenylalanine alone and only partially by tyrosine. The tyrosine requirement for nitrogen equilibrium can be covered by either tyrosine itself or by phenylalanine, which may yield it in metabolism. This interpretation receives added support from the fact that Womack and Rose were able to secure maintenance of body weight for 20 days in four rats on a diet containing tyrosine but no phenylalanine except in the vitamin addenda used.

A logical extension of these conceptions leads to the conclusion that the amino acid requirements for different species may differ, not because of qualitative differences in their synthetic capacities, but because of differences between the rate of supply and the rate of demand. Thus, on an arginine-free diet, the synthesis of arginine in the rat proceeds at such a rate that growth can occur at 75% of its normal rate. In the chick, the restriction of growth is even more severe, since even 20% of casein in the diet apparently fails to provide sufficient arginine for maximum growth (Klose, Stokstad and Almquist, '38). It seems quite probable, that the amino acids that must be present in the diet preformed to permit maximal growth would be fewer in number for a slowly-growing species, such as the human species, than for a rapidly-growing species, such as the rat.

CONCLUSIONS

For the replacement of endogenous losses of nitrogen, the adult rat does not need the following dietary amino acids: lysine, leucine, histidine, arginine, and phenylalanine. These amino acids are all required for growth in the rat.

The adult rat requires both cystine and methionine for the maintenance of nitrogen equilibrium. The cystine requirement can be covered by dietary methionine, but methionine requirement cannot be satisfied by dietary cystine.

The endogenous metabolism of the adult rat involves the destruction of tyrosine, or of tissue constituents derived from tyrosine, but not the destruction of phenylalanine. The

losses thus incurred may be replaced by either dietary tyrosine or by dietary phenylalanine.

The differences between the dietary requirements of amino acids for growth and for maintenance of nitrogen equilibrium may be satisfactorily explained on the basis of differences between rates of supply and of demand with respect to the two functions.

The adult rat may be maintained in nitrogen equilibrium on a nitrogen supply containing only the following amino acids: threonine, isoleucine, tryptophane, valine, methionine, tyrosine and norleucine.

The precise function of norleucine in maintaining the nitrogenous integrity of the tissues needs further study. It may function in promoting the synthesis of lysine or of leucine, or of both.

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THE INTERDEPENDENCE AMONG AMINO ACIDS IN THEIR UTILIZATION IN THE ENDOGENOUS METABOLISM [1]

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There is a clear distinction between the amino acid requirements for the replacement of the endogenous losses of nitrogen and those for the construction of new tissues in growth. In the latter case, the law of the minimum strictly holds: no protein synthesis occurs unless all of the essential amino acids (except arginine) are present in the food supply, while for any given food supply the rate of protein synthesis is determined by the concentration of that essential amino acid present in the smallest proportion with reference to the animal's requirements. However, in the replacement of endogenous losses of nitrogen the law of the minimum does not apply. Incomplete proteins such as gelatin and zein, that are not able to maintain nitrogen equilibrium are nevertheless utilized to a considerable extent (McCollum and Steenbock, '12), and rations containing incomplete amino acid mixtures are definitely superior to those containing no considerable amounts of nitrogen (Mitchell, '16) in the preservation of life. Mitchell and Hamilton ('29) have distinguished these two types of amino acid requirements by the terms "aggregate requirements," referring to the protein synthesis occurring during growth, and "particulate requirements," referring to the maintenance of the nitrogenous integrity of the

[1] The substance of this paper was taken from a thesis submitted by E. Wise Burroughs to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Husbandry, July, 1939.

tissues and to whatever synthetic reactions may be therein involved. The "particulate requirements" of maintenance have been compared to the requirements of inorganic ions (Mitchell, '16).

It has been shown (Burroughs, Burroughs and Mitchell, '40 a) that individual amino acids, particularly isoleucine, histidine, arginine and cystine, are well utilized in the endogenous metabolism, as are also certain incomplete amino acid mixtures. These findings merely confirm similar observations previously reported that have been briefly reviewed recently by Nielsen, Gerber and Corley ('39), all of which support the proposition of the particulate nature of the amino acid requirements for the maintenance of nitrogen equilibrium.

There is, however, other evidence indicating that these requirements are not entirely independent of each other. Elman ('39) found that the injection into dogs of an acid hydrolysate of casein plus added tryptophane and methionine induces nitrogen equilibrium. However, if the tryptophane and methionine are injected 6 hours after the injection of the casein hydrolysate, nitrogen equilibrium does not supervene. The utilization of some of the amino acids in the casein hydrolysate is evidently dependent upon the simultaneous presence in the tissues of tryptophane or methionine or both. Of similar significance are the results of some of the experiments reported in our preceding paper ('40 b). The withdrawal from the daily diet of approximately 4 mg. of nitrogen in the form either of threonine, isoleucine, tryptophane, valine or methionine occasioned average increases in the urinary nitrogen of 37.7, 24.9, 21.2, 24.1 or 16.0 mg. of nitrogen per day, respectively, with no change in the daily intake of nitrogen. Evidently here, also, the utilization in the endogenous metabolism of some amino acids is dependent upon the simultaneous presence of other amino acids in the metabolic mixture.

The experiments to be reported in this paper were designed to throw further light upon this interesting interdependence of one amino acid upon others in replacing the endogenous losses of nitrogen from adult albino rats.

EXPERIMENTAL METHODS

A series of eight amino acid mixtures (table 1) was made up containing from twelve to twenty constituents. In each mixture, the constituent amino acids contributed equal proportions of nitrogen. The mixture of twenty amino acids

TABLE 1

The percentage distribution of nitrogen in the experimental amino acid mixtures

	MIXTURE [1]							
	12	14	16	18	20	14a	16a	18a
dl-threonine	...	7.14	6.25	5.56	5.00	...	...	...
dl-isoleucine	...	7.14	6.25	5.56	5.00	...	...	...
l-tryptophane	...	...	6.25	5.56	5.00	...	...	5.56
dl-valine	...	...	6.25	5.56	5.00	...	...	5.56
dl-methionine	...	...	...	5.56	5.00	...	6.25	5.56
dl-histidine monohydrochloride monohydrate	...	...	...	5.56	5.00	...	6.25	5.56
dl-lysine monohydrochloride	...	...	...	...	5.00	7.14	6.25	5.56
dl-phenylalanine	...	...	...	...	5.00	7.14	6.25	5.56
dl-leucine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
d-arginine monohydrochloride	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-cystine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-tyrosine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
glycine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-alanine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-serine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-norleucine	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-aspartic acid	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
dl-glutamic acid monohydrate	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-proline	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
l-hydroxyproline	8.33	7.14	6.25	5.56	5.00	7.14	6.25	5.56
Total	100	100	100	100	100	100	100	100

[1] For composition of each mixture see text.

was the complete mixture described in our earlier paper ('40 b), while the mixture of twelve acids differed from the complete mixture in not containing the following constituents: threonine, isoleucine, tryptophane, valine, methionine, histidine, lysine and phenylalanine. The intermediate mixtures

were built up by adding to the mixture of twelve acids, pairs of amino acids in the above list, or by subtracting pairs of acids from the complete mixture, according to the following scheme:

Mixture no 12.   Contained the complete mixture minus the above eight amino acids.
Mixture no. 14.   Mixture no. 12 + threonine and isoleucine.
Mixture no. 16.   Mixture no. 14 + valine and tryptophane.
Mixture no. 18.   Mixture no. 16 + methionine and histidine.
Mixture no. 20.   Mixture no. 18 + lysine and phenylalanine.
Mixture no. 18a. Mixture no. 20 — threonine and isoleucine.
Mixture no. 16a. Mixture no. 18a — valine and tryptophane.
Mixture no. 14a. Mixture no. 16a — methionine and histidine.

The order in which the eight amino acids tested in this way were used was, for the five essential amino acids, roughly in proportion to the size of the negative nitrogen balances induced by their individual withdrawal from the diet. Mixture no. 18 was incomplete, because of the low proportion of tyrosine (table 3, Burroughs, Burroughs and Mitchell, '40 b).

The value of each mixture in the replacement of endogenous losses of nitrogen was determined upon either two or three adult female rats. All mixtures were fed at the same nitrogen level, approximately 80 mg. daily, as supplements to a diet containing only 3.6 mg. of nitrogen, of which each rat received 7 gm. per day. The rats were first placed upon the standardizing diet described elsewhere (Burroughs, Burroughs and Mitchell, '40 b) and then upon the low-nitrogen diet. On the second day on this diet, the amino-acid mixtures were given and were continued until the nitrogen balances were stable. The metabolism apparatus used, the method of feeding, and the methods of collecting, preserving and analyzing the excreta are described elsewhere (Burroughs, Burroughs and Mitchell, '40 b).

DISCUSSION OF EXPERIMENTAL RESULTS

The average data collected in table 2 relate only to those portions of the collection periods for which fairly constant daily nitrogen balances were secured. The collection periods were of 5 to 7 days duration.

The nitrogen balances indicate that the addition of threonine and isoleucine to mixture no. 12 did not improve its value in replacing endogenous losses of nitrogen, although the subsequent addition of valine and tryptophane and then of

TABLE 2

The average nitrogen balance data for the various experimental amino acid mixtures tested

RAT NO.	AVER-AGE BODY WEIGHT	AMINO ACID MIX-TURE TESTED	DIFFERENCE FROM PRECEDING MIXTURE	NITROGEN METABOLISM PER DAY				
				No. of days aver-aged	In-take	Urine	Feces	Bal-ance
	gm.			*days*	*mg.*	*mg.*	*mg.*	*mg.*
54	197	12	. . . .	3	82.6	86.8	13.7	—17.9
55	189	12	. . . .	5	82.6	96.9	8.5	—22.8
60	168	12	. . . .	5	82.6	89.1	12.9	—19.4
Aver.	185	12	. . . .		82.6	90.9	11.7	—20.0
56	190	14	+ threonine + isoleucine	4	82.4	99.1	9.6	—26.3
57	183	14	+ threonine + isoleucine	4	82.4	99.3	10.3	—27.2
50	229	14	+ threonine + isoleucine	4	82.4	98.7	9.9	—26.2
Aver.	201	14	+ threonine + isoleucine		82.4	99.0	9.9	—26.5
58	181	16	+ valine + tryptophane	4	83.2	81.2	12.4	—10.4
59	180	16	+ valine + tryptophane	4	83.2	83.0	9.6	— 9.4
51	219	16	+ valine + tryptophane	4	83.2	84.3	9.2	—10.3
Aver.	193	16	+ valine + tryptophane		83.2	82.8	10.4	—10.0
61	176	18	+ methionine + histidine	4	83.3	86.8	9.8	—13.3
65	157	18	+ methionine + histidine	4	83.3	64.6	28.5[1]	— 9.8
66	218	18	+ methionine + histidine	4	83.3	84.9	11.7	—13.3
Aver.	184	18	+ methionine + histidine		83.3	78.8	16.7	—12.2
52	214	20	+ lysine + phenylalanine	5	83.4	63.6	7.7	+12.1
53	211	20	+ lysine + phenylalanine	5	83.4	64.4	6.8	+12.2
60	172	20	+ lysine + phenylalanine	5	83.4	61.2	11.4	+10.8
Aver.	199	20	+ lysine + phenylalanine		83.4	63.1	8.6	+11.7
71	162	18[1]	— threonine — isoleucine	4	82.9	104.0	10.8	—31.9
72	174	18[1]	— threonine — isoleucine	5	82.9	104.6	9.8	—31.5
Aver.	168	18[1]	— threonine — isoleucine		82.9	104.3	10.3	—31.7
69	165	16[1]	— tryptophane — valine	4	82.6	103.6	7.2	—28.2
70	182	16[1]	— tryptophane — valine	4	82.6	103.4	11.7	—32.4
Aver.	173	16[1]	— tryptophane — valine		82.6	103.5	9.4	—30.3
67	158	14[1]	— methionine — histidine	6	81.4	100.9	9.8	—29.3
68	189	14[1]	— methionine — histidine	6	81.4	108.5	8.5	—35.6
Aver.	173	14[1]	— methionine — histidine		81.4	104.7	9.1	—32.4

[1] Diarrhea.

methionine and histidine decreased the net losses of nitrogen to about half those incurred on mixture no. 12. Furthermore, the withdrawal of threonine and isoleucine from the complete mixture was associated with a marked negative balance of about 32 mg. of nitrogen daily, a level that was not exceeded by subsequent withdrawals first of tryptophane and valine and then of methionine and histidine.

The general picture presented by these balance data indicates that the presence of threonine or isoleucine, or possibly of both, in the diet is necessary for the utilization of the other amino acids essential for nitrogen equilibrium. Also, since the nitrogen balances for rats of the sizes used in these tests (170 to 200 gm.) while subsisting on a nitrogen-free diet would range from about —45 to —55 mg. daily, it appears that some 30 to 50% of these losses can be covered by a variety of incomplete combinations of amino acids, even by mixtures containing none of those acids whose presence in the diet is essential for the attainment of nitrogen equilibrium.

CONCLUSIONS

Some 30 to 50% of the nitrogen lost in the endogenous catabolism may be replaced from a variety of incomplete dietary combinations of amino acids, even by mixtures containing none of those amino acids whose presence in the diet is essential for the attainment of nitrogen equilibrium. This much of the nitrogen requirement for the maintenance of the integrity of the tissues is thus apparently an undifferentiated one, that requires for its complete satisfaction no specific amino acids.

The remainder of the requirement not only relates to specific amino acids, but also is of such a nature that the utilization of the specific amino acids required depends upon the simultaneous presence in the diet of certain combinations of the essential amino acids. Of this interdependence in the requirements of the essential amino acids, it can be concluded, from the experiments reported in this paper, only that threonine and isoleucine, either individually or together, limit the

utilization of the other essential amino acids and possibly occupy a key position in the anabolism consequent upon the endogenous disintegration of tissue constituents.

Thus, the endogenous losses of nitrogen in the adult animal seem to result from the destruction in the tissues of many types of nitrogenous constituents of relatively simple structure, rather than of complex amino-acid aggregates, such as the tissue proteins.

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FACTOR II DEFICIENCY IN DOGS

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Fouts, Helmer, Lepkovsky and Jukes ('38) found that puppies maintained on synthetic casein diet supplemented with vitamin B_1, riboflavin and rice-polish extract containing factor I (vitamin B_6) died with symptoms of blacktongue. Puppies receiving, in addition, purified liver extract containing chick antidermatitis factor (factor II) and nicotinic acid showed normal rate of growth. Although Elvehjem, Madden, Worley and Strong ('37) cured blacktongue by administration of nicotinic acid amide prepared from a similar liver extract, Lepkovsky and Jukes,[1] Dann ('37), Cook, Clarke and Light ('37), and Mickelsen, Waisman and Elvehjem ('38) were unable to cure chick dermatitis by administration of nicotinic acid. It is evident, therefore, that the purified liver extract contains at least two active substances, one of which (nicotinic acid) cures blacktongue in dogs and pellagra in humans, and the other (chick antidermatitis factor) cures pellagra-like symptoms in chicks. The purpose of the present experiment was to study in dogs the deficiency state produced by lack of the factor or factors contained in the purified liver extract other than nicotinic acid.

METHODS

Mongrel adult dogs weighing from 7.1 to 9.4 (average 8.2) kilos were used. Dogs 5, 6 and 7 had been studied during one or more periods of factor I deficiency before the present experiment was started. The synthetic diet, however, had been

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[1] Cited as a personal communication in the paper by Fouts, Helmer, Lepkovsky and Jukes ('37).

completely supplemented for at least 1 month before the purified liver extract was excluded from the supplements. The basic diet consisted of the following: washed casein 41.4 gm., sucrose 29.6 gm., Crisco 25.7 gm., bone ash 2.0 gm., and salt mixture no. 185 (McCollum and Simmonds, '18) 1.3 gm. The dogs were allowed to eat as much of the diet as desired. Dogs 1, 2, 3 and 4 received daily by dropper thiamin chloride 50 mcg. per kilo, synthetic riboflavin 60 mcg. per kilo, nicotinic acid 2 mg. per kilo, 0.3 cc. per kilo of rice-polish extract K-85E or 0.6 cc. K-110G per kilo, in addition to vitamins A and D. Dogs 5, 6 and 7 received the same amounts of the above supplements except that 60 mcg. of crystalline factor I per kilo (Lepkovsky, '38) was substituted for the rice-polish extracts. Dogs 5 and 7 received, in addition, 0.5 gm. iron and ammonium citrate and 0.5 mg. copper sulfate daily. The rice-polish extracts containing factor I free from factor II and the purified liver extract (fed to dog 2) were similar to those used previously (Fouts, Helmer, Lepkovsky and Jukes, '38). Red blood cell counts, hemoglobin determinations (Newcomer) and white blood cell counts were made at weekly intervals. Hematocrit measurements were made by use of Wintrobe tubes.

RESULTS

Six of the seven dogs increased in weight after being fed the diet. Dog 3 gained most, namely, 3.7 kilos. Following the preliminary gain in weight, all lost weight (4, 3, 1.9, 5.3, 6, 4, and 4.1 kilos respectively). Loss of weight was associated with progressive decrease in appetite. All animals developed diarrhea within 7 to 66 days; it was intermittent in character but became severe and often bloody before death. Decrease in appetite was especially marked during exacerbations of diarrhea. Vomiting occurred frequently. As weight decreased they became quiet and were very weak shortly before death. The three short-haired dogs (nos. 3, 6 and 7) developed ulcers of the skin over the shoulders, neck and back. The ulcers were superficial, varied in size and number, had a punched-out appearance, bled occasionally, and all showed varying degrees

of healing at time of death of the dogs. Thinning of hair on the head and inner aspects of extremities occurred in dogs 5, 6 and 7. There appeared to be a slight increase in graying of the hair about the mouth in dogs 6 and 7.

The blood findings are presented in tables 1 and 2. Anemia occurred in six of the seven dogs studied. Dog 1 developed a slight anemia by the 252nd day (red blood cell count 5.04 million, hemoglobin 13.4 gm., and hematocrit 38 cc.). Administration of 50 gm. of glucose daily and addition of 6 gm. of sodium chloride to the supplements were followed by a definite but temporary improvement in the general condition and decrease in diarrhea. This improvement was associated with definite hydraemia as demonstrated by a rapid fall in red blood cell count, hemoglobin, hematocrit, blood urea, and serum proteins. At death 36 days later, the red blood cell count had increased to 5.29 million and the hemoglobin to 12.3 gm. Dogs 2 and 4 had more severe anemia than the others (red blood cell counts 4.08 million and 3.67 million, respectively; hemoglobin 11.5 gm. and 8.8 gm.). These dogs did not eat the diet as well as the others and the survival time was shorter. Dog 3 had a slight anemia at the beginning of the studies. This animal gained 3.7 kilos during the first 139 days on the diet. During the same period there was an increase in the red blood cell count and the percentage of hemoglobin. Anemia did not recur although the weight decreased after the 139th day. The erythrocyte counts before death of the three dogs (5, 6 and 7) receiving only the four crystalline supplements were 5.02, 4.87 and 4.93 million, respectively, and the corresponding hemoglobin values 11.0, 14.8 and 12.3 gm. per 100 cc.

Serum proteins decreased in all the dogs examined. There was no increase in serum potassium or consistent fall in plasma chlorides. A terminal rise in blood urea occurred in dogs 4 and 7. The terminal rise in blood urea and decrease in plasma chlorides in dog 7 might have been related to the extensive pneumonia found at autopsy.

Survival on the diet varied between 197 and 289 days (average 243 days) except for two dogs receiving vitamin B_6

TABLE 1

Blood findings in dogs 1, 2, 3 and 4

DOG	DAYS ON DIET	RED BLOOD CELLS	HEMO- GLOBIN	WHITE BLOOD CELL COUNTS	HEMATO- CRIT	MEAN CORPUS- CULAR VOLUME	REMARKS
		millions /cu.mm.	*gm. %*	*per cu.mm.*	*cc./100 cc.*	*cubic μ*	
1	0	7.47	15.3	14,300	51.0	68.3	
	131	6.57	16.3	8,200	50.0	76.1	
	247	5.61	15.6	5,300			
	252	5.04	13.4	5,600	38.0	76.0	
	255	4.35	11.6	17,450	31.0	71.7	
	258	3.59	9.7	15,650	25.0	69.6	
	261	3.43	8.4	5,800			
	272	4.43	11.0	5,100			
	287	5.29	12.3	7,350			
	289						Dead
2	0	7.41	14.3	15,450	47.0	63.4	
	10	7.40	14.2				Purified liver extract (4 cc. per kilo)
	35	5.44	12.5	30,750			
	63	7.41	15.0	11,250			
	65						Purified liver extract discontinued
	112	6.89	15.8	7,350			
	135	4.19	14.4	22,350	31.0	74.0	
	147	4.08	11.5	38,650			
	148						Dead
3	0	5.90	11.9	9,500	41.0	69.5	
	49	6.22	14.9	7,650			
	70	6.14	17.0	7,650			
	77	7.61	18.4	6,600			
	118	5.03	13.9	5,100			
	135	6.27	15.3	7,750	42.5	67.8	
	146	4.80	15.2	8,250			
	168	5.98	18.0	7,450			
	182	6.92	17.2	8,050			
	189	5.74	16.6	8,500			
	196	6.66	18.6				
	197	8.83	24.3	8,050			Dead
4	0	7.51	15.0	12,500	50.0	66.6	
	28	8.20	18.8	30,750			
	49	5.52	11.6	39,450			
	70	4.36	11.7	6,450	31.0	71.1	
	86	3.67	8.8	7,800	26.0	70.8	
	87						Dead

TABLE 2

Blood findings in dogs 5, 6 and 7

DOG	DAYS ON DIET	ERYTHROCYTE COUNT IN MILLIONS	HEMOGLOBIN IN GM.%	WHITE CELL COUNT	HEMATOCRIT IN CC./100 CC.	MEAN CORPUSCULAR VOLUME IN CUBIC μ	UREA NITROGEN IN MG.%	TOTAL PROTEIN IN GM.%	ALBUMIN IN GM.%	GLOBULIN IN GM.%	PLASMA CHLORIDE IN MG.%	SERUM POTASSIUM IN MG.%	GLUCOSE IN MG.%
		per cu.mm.		*per cu.mm.*									
5	−7	5.85	13.3	4,650	40	68.4							
	0	6.45	12.6	5,650			17.2					18.0	56
	84						15.5	4.70	2.64	2.06	389		57
	91	6.44	15.8	8,950	42.5	66.4							67
	100	5.66	15.0	7,900									
	112						20.0	4.75	2.58	2.17	362		70
	114	6.05	16.0	7,300	44.0	72.7	32.7	4.59	2.32	2.27	369		93
	133						25.9	4.76	1.96	2.80	377		103
	142						26.2	4.70	2.04	2.66	381		100
	149	4.86	16.8										
	154						13.0	3.86	1.64	2.22	372		111
	156						11.5	4.00	1.91	2.09	356		104
	170	5.87	13.1	10,400	36.0	61.3							
	175						12.9	4.34	1.89	2.45	351		90
	177						17.8	4.35	1.66	2.69	358		100
	198	5.08	13.6	7,500	36.0	70.8	15.7	4.30	1.61	2.69	354		120
	205						14.2	4.48	1.86	2.62	350		144
	212	4.76	11.1										
	217	5.02	11.0	13,150	33.0	69.3	12.1	3.98	2.18	1.80		18.0	118
	219												
	221												Dead
6	0	7.44	16.8	7,950	44.0	63.2							61
	93	6.96	15.5	3,450			14.3						53
	204												63
	209	6.16	15.3	5,550	44.0	73.8	19.8	5.75	3.12	2.63	383	21.0	
	218	6.36	17.0	5,100									68
	224												59
	225	6.37	16.5	5,350									
	230	4.47	14.9	33,950			13.5	5.74	3.36	2.38	379		
	251	4.62	12.8	40,200	35.0	78.3	10.0	5.94	3.03	2.91	382		114
	252	4.87	14.8	4,300	33.5	72.5	18.5	5.11	2.63	2.48	329	22.0	
	257												Dead
7	−8	7.61	14.8	8,800	47.0	61.8							
	0	7.78	16.6	5,150			17.2						43
	93	8.17	18.4	7,900	51.0	62.4						15.3	46
	204												63
	209	6.60	15.8	9,000	46.0	69.7	12.7	4.98	2.36	2.62	369		45
	218												
	224	5.89	14.5	29,000			12.8	5.45	2.55	2.90	390		
	225												
	230	5.54	13.4	16,400			16.4	5.50	2.38	3.12	387		48
	232												
	250	4.93	12.3	5,500			157.0	4.91	1.89	3.02	317		83 Dead

supplied as a component of the rice-polish extract. These dogs died on the eighty-third and eighty-seventh day. They showed loss of appetite within 4 to 7 days. One animal (dog 2) stopped eating and developed a diarrhea on the seventh day. Factor II (liver extract CF I, 4 cc. per kilo) was added to the supplements given from the tenth to the sixty-third day. All symptoms slowly disappeared and body weight gradually increased. When the liver extract was discontinued the symptoms recurred within 47 days and the dog died within 83 days. The other animal ate very little from the start, its food intake averaging 47 gm. per day. Survival of the three dogs receiving the four crystalline supplements varied between 221 and 257 days.

At autopsy the liver of each dog appeared to be fatty. One animal had superficial ulcerations of the gums, another had numerous shallow ulcers and hemorrhages in the ileum, and dog 6 had a penetrating ulcer on the lesser curvature of the stomach as well as numerous superficial ulcers scattered throughout this organ and the first portion of the duodenum. The bone marrow of dogs surviving for long periods of time showed increased cellularity. Dogs 2 and 7 had extensive pneumonia.

DISCUSSION

The deficiency disease which developed in dogs subsisting upon a synthetic casein diet supplemented with thiamin chloride, synthetic riboflavin, nicotinic acid, and rice-polish extract containing factor I or crystalline factor I, was similar to the deficiency state produced by Chick, Macrae, Martin and Martin ('38) in pigs fed a synthetic diet similarly supplemented. Central nervous system involvement observed in factor II deficiency by Chick, Macrae, Martin and Martin ('38) and by Wintrobe, Mitchell and Kolb ('38) in pigs, and by Phillips and Engel ('39) in chicks, was not observed.

The anemia noted in six of the seven dogs was similar to that found by Chick, Macrae, Martin and Martin ('38) and Wintrobe, Sawter and Lesco ('39) in swine. It was of moderate severity and there tended to be an increase in mean cor-

puscular volume. The decrease in serum proteins which occurred in all the dogs examined might account for this tendency (Bethell, '36). The two dogs which ate the least amounts of the diet had the most severe anemia at death. Study of blood electrolytes revealed no evidence suggesting adrenal insufficiency. These findings do not substantiate those of Morgan and Simms ('39) who found striking and constant atrophy of adrenals in rats deprived of filtrate factor or factors. The ulcers of the skin in the short-haired dogs, however, are similar to those reported by these authors.

There appeared to be no difference in the symptoms of the deficiency states in dogs receiving the rice-polish extract and those receiving the crystalline factor I except that two of the dogs receiving the rice-polish extract succumbed in a shorter time. The survival time of the other five dogs was fairly constant. It would appear that the previous dietary history must have influenced the time of development of symptoms and survival time. Dog 2 developed symptoms within 7 days when first started on the deficient diet but did not show any symptoms for 47 days after having received purified liver extract for 53 days. The three dogs receiving similar amounts of purified liver extract for long periods of time (163 to 619 days) previous to this experiment survived from 221 to 257 days. Coprophagy probably influenced the survival time but it did not appear to be the determining factor inasmuch as when it occurred it was not observed until late in the study and after symptoms of the deficiency had developed.

SUMMARY

1. Adult dogs fed a synthetic casein diet, supplemented with thiamin chloride, riboflavin, nicotinic acid, and crystalline factor I (vitamin B_6), and apparently deficient only in a factor or factors contained in a purified liver extract other than nicotinic acid, developed a deficiency state characterized by loss of appetite, marked loss of weight, intermittent diarrhea, moderate anemia, and death.

2. Short-haired dogs showed ulcerations of the skin while long- or wire-haired dogs did not.

3. It was not determined whether the deficiency state described is due to lack of factor II (chick antidermatitis factor) alone or to the lack of this and other as yet unisolated components of the vitamin B complex.

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THE RETENTION OF CALCIUM AND PHOSPHORUS BY PRE-SCHOOL CHILDREN [1]

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ONE FIGURE

The feasibility of supplementing the diets of children with certain vitamins and minerals has been demonstrated by Aykroyd and Krishnan ('38), Privitera ('38), Schlutz and his associates ('38), Summerfeldt and Ross ('38), and Tisdall et al. ('30). From past experience it is known that the utilization of minerals is influenced by the source. Thus, the question arises as to the availability of added substances in fortified foods for use by the human body.

Several studies of the calcium metabolism of children have been made in recent years (Daniels et al., '34, '35; Outhouse et al., '39; Kinsman and associates, '39). A survey of the available data shows that marked variations in calcium retention have been noted. Furthermore, individual retentions differ materially from time to time in the same child. Fairbanks and Mitchell ('36) have shown with growing rats that calcium requirements can be determined only when the body stores have been saturated by appropriate pre-feeding.

Several investigations comparing the availability of calcium and phosphorus from milk and from inorganic sources have

been made. A study of these data, along with their own, led Henry and Kon ('39) to make the following statement, "As far as they go, these studies appear to indicate that if there is a difference between the availabilities of calcium from milk and from inorganic sources it cannot be very marked and our metabolic experiments confirm such a view."

The object of this study has been to determine whether the calcium of an alkaline salt mixture added to a wheat farina would be as well utilized by pre-school children as an equivalent amount of this element supplied by milk. After adequate pre-feeding to insure saturation with calcium and phosphorus, part of the milk of the basal diet was omitted, and the calcium in this quantity of milk was replaced by an equivalent amount furnished by the salt mixture[3] in the fortified cereal.

EXPERIMENTAL

Ten children, six boys and four girls, whose ages, weights, and heights at the beginning and end of the experiment are shown in table 1, were selected to serve as subjects. A routine was established to make a program as nearly normal as possible within the rigid control required. In inclement weather, the children received exposure to rays from an ultraviolet lamp for 15 minutes daily.

The diets were similar to those used by Daniels et al. ('34). A typical diet for one subject throughout the study is illustrated in table 2. The basal diet was planned so that approximately 50 mg. of calcium per kilogram body weight per day or 80% of the total ingested was supplied by milk and the remainder by the solid food. During experimental periods, 200 mg. of milk calcium were replaced by 200 mg. of calcium in the fortified cereal. The food was supplemented with 1800 U.S.P. units of vitamin D, 12,000 U.S.P. units of vitamin A, and 300 U.S.P. units of thiamin-hydrochloride daily. Distilled water was used for drinking and cooking.

The children were divided into two groups as evenly matched as possible with regard to weight and age. These two groups

[3] A mixture of calcium, sodium and iron phosphates.

TABLE 1

Growth record of children

Subject	Group	Sex	BEGINNING			END					Expected gain [1]	
			Age	Weight	Height	Age	Weight	Height	Gain in weight	Gain in height	Weight	Height
			yrs. mos.	kg.	cm.	yrs. mos.	kg.	cm.	kg.	cm.	kg.	cm.
1	LP	F	5 – 10	16.3	102.2	6 – 4	18.5	105.4	2.2	3.2	1.1	4.1
2	LP	F	4 – 2	16.2	101.0	4 – 8	18.4	105.4	2.0	4.4	1.1	3.4
3	PL	M	5 – 0	18.5	102.9	5 – 6	20.3	108.6	1.8	5.7	0.7	3.5
4	PL	M	5 – 4	18.0	106.7	5 – 10	20.4	111.1	2.4	4.4	0.2	2.5
5	PL	F	2 – 11	14.5	95.3	3 – 5	17.0	98.4	2.5	3.1	1.0	3.6
6	LP	M	3 – 7	17.6	109.5	4 – 1	19.6	113.7	2.0	4.2	0.5	2.6
7	LP	M	5 – 5	17.7	99.7	5 – 11	19.1	104.8	1.4	5.1	0.7	2.6
8	LP	M	5 – 4	20.1	114.3	5 – 10	22.9	120.0	2.8	5.7	1.1	4.1
9	PL	F	4 – 2	15.5	99.1	4 – 8	17.3	103.5	1.8	4.4	0.9	3.5
10	PL	M	4 – 0	14.9	94.6	4 – 6	17.0	97.8	2.1	3.2		

[1] Based on data at Iowa Child Research Station ('29).

TABLE 2

Sample diets. Child no. 2 in group 1. Figures are in grams or cubic centimeters unless noted differently

	PERIOD 1	PERIOD 2	PERIOD 3	PERIOD 4	PERIOD 5
Breakfast					
Oatmeal	10	10	10	10	—
Cream of Wheat	—	—	—	—	15 New
Sugar	7	7	9	7	7
Milk	225	225	162	225	225
Bread	15	15	15	15	15
Butter	5	5	5	5	8
Jelly	10	10	10	10	10
Prunes	40	40	40	40	40
Egg	50	50	50	50	50
Mid A.M.					
Orange juice	120	120	120	120	120
Vit. B tablets	1 tablet	1 tablet	1 tablet	1 tablet	1 tablet
Oleum Perco. (vit. D)	10 drops	10 drops	10 drops	10 drops	10 drops
Dinner					
Cream of Wheat	—	—	—	—	10 New
Beef	30	30	30	30	30
Potato	85	85	85	85	85
Beans	40	—	—	40	—
Carrots	—	40	40	—	40
Bread	15	15	15	15	15
Butter	10	10	10	10	10
Milk	20	20	20	20	20
Tomato juice	110	110	110	110	110
Doughnut	—	—	23	—	—
Mid P.M.					
Banana	70	70	70	70	70
Milk	218	218	—	218	50
Brazil nuts	—	—	14	—	—
Supper					
Cream of Wheat	15 Old	15 Old	15 Old	15 Old	15 New
Sugar	7	7	9	7	7
Milk	225	225	162	225	225
Bread	15	15	15	15	15
Butter	5	5	5	5	9
Jelly	10	10	10	10	10
Apple sauce	60	60	60	60	60
Chocolate	15	15	15	15	15
Ca lactate	2.856*	—	—	—	—
Triple PO₄	—	—	—	1.213*	—

* Equivalent to 256 mg. calcium.

are designated as LP and PL, respectively: the former being saturated with calcium from lactate in the first and calcium from phosphate in the second saturation period. PL children were saturated with calcium from phosphate in the first and calcium from lactate in the second saturation period. The experiment was divided into five metabolism periods as shown in table 3.

TABLE 3

Diet periods

GROUP	PERIOD 1 6 WEEKS	PERIOD 2 5 WEEKS	PERIOD 3 2 WEEKS	PERIOD 4 4 WEEKS	PERIOD 5 6 WEEKS
LP	Basal diet plus 200–256 mg. Ca from lactate	Basal diet	Ca intake reduced to approximately ⅔ that of basal diet	Basal diet plus 256 mg. Ca from triple phosphate	Basal diet except 200 mg. milk Ca replaced by 200 mg. Ca supplied by fortified farina
PL	Basal diet plus 200–256 mg. Ca from triple phosphate	Basal diet except 200 mg. milk Ca replaced by 200 mg. Ca supplied by fortified farina	Ca intake reduced to approximately ⅔ that of basal diet	Basal diet plus 256 mg. Ca from lactate	Basal diet

To rule out variations which might be assigned to changes of weather, season and age, the periods for the PL group were reversed and the sequence was 4, 5, 3, 1, 2.

The dietary routine was interrupted for a few days between periods, care being taken that the calcium intake remained the same as that of the preceding period.

DISCUSSION OF RESULTS

Calcium. By referring to table 4 and figure 1, it may be seen that retention during the first two periods was higher than that noted during the last three. It has been noted in other studies of this nature that previous depletion due to inadequate mineral intake or poor utilization may be responsible for early high retentions. In period 1 children (LP), being saturated with the lactate, retained more calcium than those obtaining this element from the phosphate mixture. Using the data for

TABLE 4

Calcium and phosphorus metabolism

PERIOD	GROUP	CALCIUM							PHOSPHORUS						CA:P[1]	CA:P FOOD	WEEKLY STOOL WEIGHT	NITROGEN RETENTION PER KG. PER DAY
		Weekly intake	Weekly output			Weekly retention		Ca per gram stool	Weekly intake	Weekly output			Weekly retention					
			Amount	Feces	Urine					Amount	Feces	Urine						
		gm.	*gm.*	%	%	*gm.*	%	*mg.*	*gm.*	*gm.*	%	%	*gm.*	%			*gm.*	*mg.*
1	LP[2]	9.19	7.08	95.9	4.1	2.11	22.9	10.1	8.72	7.37	42.7	57.3	1.35	15.5	1.58	1.05	682	
1	PL[3]	9.17	7.57	95.2	4.8	1.60	17.4	11.1	9.80	8.52	38.2	61.8	1.28	13.2	1.21	0.94	657	
2	LP	8.14	6.43	93.3	6.7	1.71	21.1	10.1	8.46	7.63	44.6	55.4	0.83	10.4	2.04	0.96	580	61.2
2	PL	7.90	5.99	91.5	8.5	1.91	24.1	9.7	8.43	7.33	35.5	64.5	1.10	13.1	1.75	0.94	571	53.6
3	LP	5.01	4.06	90.0	10.0	0.95	18.9	6.8	7.33	6.68	43.4	56.6	0.65	8.8	1.66	0.68	542	
3	PL	4.73	3.90	88.2	11.8	0.83	17.7	6.4	7.06	6.50	39.7	60.3	0.56	8.1	1.48	0.67	547	
4	LP[3]	9.69	8.24	95.0	5.0	1.45	15.0	12.2	9.94	9.29	48.1	51.9	0.65	6.8	2.18	0.97	640	55.0
4	PL[3]	9.32	7.91	92.7	7.3	1.41	15.1	10.6	8.16	7.49	38.2	61.8	0.67	8.6	2.06	1.14	692	65.0
5	LP	8.12	6.62	93.3	6.7	1.50	18.5	11.0	8.80	7.78	43.0	57.0	1.02	11.5	1.49	0.92	563	51.9
5	PL	7.74	6.29	91.8	8.2	1.45	18.7	10.3	8.41	7.54	36.5	63.5	0.89	10.4	1.65	0.92	566	53.1
Av.	LP	8.03	6.49	93.5	6.5	1.54	19.3	10.9[4]	8.64	7.75	44.4	55.6	0.90	10.6	1.79	0.92	616	56.0
Av.	PL	7.77	6.33	91.9	8.1	1.44	18.6	10.4[4]	8.37	7.48	37.6	62.4	0.90	10.7	1.63	0.92	621	57.2

[1] Calculated on basis of milligrams retained per kilogram body weight per day.

[2] Calcium lactate used for saturation.

[3] Tri-calcium phosphate used for saturation.

[4] Data of period 3 omitted in computing average.

Wet ashing urine, feces and foods: Gerritz ('35).

Calcium: Shohl and Pedley's ('22) modification of McCrudden's ('09–'10; '11–'12) method.

Phosphorus: Mackay and Butler's modification of Mathison's method, Peters and Van Slyke ('32).

Total nitrogen: Kjeldahl method.

period 2 as a base, an equivalent of all of the calcium added and more was excreted in period 1 by both groups. Since amounts of calcium equivalent or more than equivalent to those added were excreted in the period of saturation, as compared with those where basal diets alone were used, it may be assumed that the basal diets contained adequate calcium for the needs of these children. In period 2, when the calcium intake was reduced and a comparison made of the availability of calcium derived from milk and from the fortified cereal, the latter source proved to be the better one from the standpoint of retention.
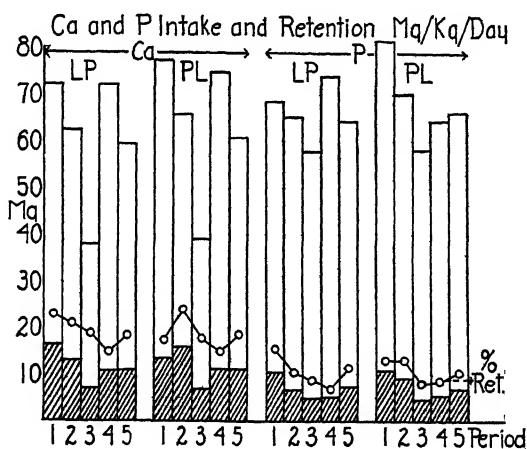
Figure 1

In period 3 the calcium-phosphorus ratio of the food, instead of being approximately 1 as in other parts of the study, approached a value of 0.7. This in itself may offer an explanation of the lowered retention. The percentage of calcium excreted in the stools, however, reached its lowest point suggesting that the disproportionate amount of phosphorus in the diet had not caused any unusual precipitation of calcium in the intestine. An adequate intake of vitamin D, however, tends to eliminate unfavorable effects due to low calcium-phosphorus ratios, and our diets contained plenty of this vitamin. Further, Sherman and Hawley ('22), using diets with low calcium-phosphorus

ratios over short periods of time, obtained data which indicated no disturbance of calcium retention.

It is interesting to note that the calcium intakes and retentions in period 3 of this study are in close agreement with those of Outhouse and her associates ('39). In the last two periods with higher intakes, the amounts retained are similar to those found by Sherman and Hawley ('22), while in the first two periods the retentions are higher. The maximum retentions did not approach those reported by Stearns and Jeans ('34). To obtain maximum retentions under the conditions of this experiment, the amount of calcium in the diet had to be greater than that suggested by Outhouse and associates ('39), but these investigators have pointed out that their data may not apply to all pre-school children. In regions of the country where there is little sunshine and where the outdoor activities of children are limited by the severity and length of the winter, it may be necessary to furnish more calcium than in regions having a more moderate climate. From our results one would conclude that the old standard of 1 gm. of calcium per day would more nearly fulfil the needs of the pre-school child than smaller amounts.

The retentions were higher in the fourth than in the preceding period and there was no significant difference between the results obtained with the two salts. Less calcium was retained by each group than in the first period. Increasing the amount of calcium resulted in a better retention of this element, but not of phosphorus (Tisdall and Drake, '38). In period 5 retentions were the same regardless of the source, showing that calcium of the fortified cereal was just as satisfactory in fulfilling the needs of the pre-school child as an equivalent amount from milk. This satisfactory utilization of calcium from an added salt is in agreement with that found by Stearns and Jeans ('34). Since the salt mixture used for fortification contained disodium phosphate, it is of interest to note that Henry and Kon ('39) found that the addition of this salt to a milk diet increased the absolute retention of calcium

and phosphorus by rats. The calcium-phosphorus ratios of food ingested approximated 1:1 as in our diets.

If the calcium output during period 5 is compared with that of period 4, it is seen that the differences between the total excretions correspond almost exactly with the extra amounts added for saturation. This affords further confirmation of our previous assumption that the basal diets were adequate in their calcium content. Since the retentions by both groups of children were essentially the same in periods 4 and 5, it is evident the children were saturated with this element.

Statistical treatment of the data for calcium retention reveals no significant differences between the two groups except in period 2. During this time, children receiving the diet in which part of the milk calcium had been replaced by calcium from the fortified cereal retained more calcium than members of the other group. Although the retentions in period 1 appeared to favor lactate as a better source of calcium than phosphate, the observed difference proved to be insignificant.

Phosphorus. The amounts stored by the children were larger at the beginning than toward the end of this investigation. This raises the question, as with calcium, as to the effect of previous depletion upon the extent of storage. The presence of more phosphorus in the diet of the PL than in that of the LP children did not increase the retention of the former group. This observation confirms that of Wang and her associates ('29) who found that increasing the amount of phosphorus in the diet over and above requirements merely caused a larger excretion in urine and feces. During the first period, when PL children were being saturated by use of the phosphate, about 12% of the phosphorus of the added salt was retained. Quite a different result was obtained with the other children (LP) while they were being saturated with the phosphate in period 4, for none of the added phosphate phosphorus was retained; in fact an equivalent of all of the added phosphorus and more was excreted during this period. Accordingly, it may be assumed that the basal diets contained sufficient phosphorus to meet the requirements of these children. The amounts re-

tained in period 1 were alike for each group, showing that the quantity added had no effect upon retention. The data for period 2 show a better retention of phosphorus when part of the milk was replaced by fortified cereal. Admittedly, the intake per kilogram body weight per day was higher for PL children who retained the larger amount of phosphorus. Yet, with a more marked difference in the daily intake per unit of body weight in the first period, no such difference in retention was found.

The girls in each group had a lower percentage output of phosphorus in the urine than did the boys. The question may be raised as to whether this finding might be due to the urinary contamination of the feces by the girls. Extreme care was used in an effort to avoid this, and the girls always were required to empty the bladder before going to stool. The nurse in charge then watched the pails to see that no urine had been passed.

Stearns ('31) has discussed the significance of the retention ratios of calcium and phosphorus in children. A survey of the literature dealing with the calcium and phosphorus metabolism of children reveals that the retention ratios vary within wide limits over a period of time even with the same child. Our data confirm this observation, but ratios obtained by us are much higher than those listed by Stearns ('31). Our average retention ratios during 6 months were 1.79 for LP and 1.63 for PL children. These values lie within the range, namely, between 1.5:1 and 2:1, recommended by Stearns.

The calcium-phosphorus ratios of bones of children as determined by several workers show some degree of variation, the values ranging from 1.9 to 2.26. In computing the amount of phosphorus required for converting retained calcium into bone, the selection of the proper ratio is important if a correct estimate is to be made of the amount of phosphorus remaining for synthesis of soft tissues. If we accept the higher ratio of 2.26 as correct, then with any ratio lower than this, phosphorus would be available for building soft tissue. We could conclude then that at all times during this study each group was building bone and soft tissue. Our data tend to confirm the idea that

in given periods some tissues grow at a more rapid rate than others, thus requiring different amounts of phosphorus. It is interesting to point out that during period 4 when high retention ratios were found, the weekly increase in body weight of children of both groups was smaller than in any other period.

The average daily retentions of phosphorus in milligrams per kilogram body weight for the 6 months were 6.9 for the LP and 7.4 for PL children. Both of these values are slightly lower than the average daily value of 8 mg. per kilogram body weight obtained by Sherman and Hawley ('22). The average retentions found by Daniels et al. ('35) also are somewhat higher than those obtained in this study.

As with calcium, statistical treatment of the data showed no significant differences between the amounts of phosphorus stored by the two groups except in period 2. Here children (PL) on the diet in which part of the milk calcium had been replaced by calcium from the fortified cereal retained significantly more phosphorus than the other children.

Nitrogen balances were determined for 1 week in periods 2, 4, and 5, and all children were in positive balance (table 4). The highest and lowest retentions found during period 2 amounted to 93 and 41 mg. per kilogram body weight per day respectively; in period 4, to 92 and 22 mg.; and in period 5, to 64 and 37 mg. The few values obtained revealed that no one child consistently retained a large or a small amount of nitrogen. In general the retentions were somewhat lower than those found by Daniels et al. ('35).

By referring to data in table 4 it is seen that there is no evidence of any laxative effect of the salt mixture used for fortification. The average weekly weights of stools of the two groups are in close agreement throughout the study with the possible exception of period 4, in which a higher value was noted with children who were receiving calcium lactate.

To gain information as to the possible effects of the triple phosphate mixture upon acid-base balance, titratable acidity (Folin, '05) of the urine was determined during period 5. Here again there was no significant difference for the two groups,

the average daily titration expressed in cubic centimeters of N/10 alkali being 156 for the LP and 168 for the PL group. These values are in agreement with those of Hawks et al. ('37).

SUMMARY AND CONCLUSIONS

A comparison has been made between milk and a fortified cereal as sources of calcium and phosphorus for pre-school children. The subjects were four girls and six boys ranging in age from 3 to 6 years. To rule out possible variations which might be due to changes in age, weather and season, the children were divided into two groups each consisting of two girls and three boys and the sequence of diet periods was reversed.

An adequate basal diet was used. To eliminate effects of previous calcium depletion, if any, extra calcium for saturation was fed in the form of lactate or the phosphate mixture used for fortification of the cereal. Final analysis shows that there is no significant difference between the retentions of calcium and phosphorus regardless of the salt used.

With the same basal diet the retention of calcium and phosphorus was determined when 200 mg. of milk calcium was replaced by 200 mg. of calcium in the fortified cereal. In one period the storage of calcium and phosphorus was significantly better when these two elements were supplied in part by the fortified cereal rather than by milk, while in a second period of comparison the availability of calcium and phosphorus as measured by retention was the same.

The children were in positive calcium, phosphorus and nitrogen balance throughout the study and more phosphorus was stored than was required to convert retained calcium into bone.

The averages of the results obtained for two groups of children over a period of 6 months were in close agreement. The daily storage of calcium was between 11 and 12 mg. and of phosphorus between 7 and 8 mg. per kilogram body weight. About 19% of the calcium and 11% of the phosphorus were retained. All children grew at rates better than those given as normal by the Iowa Child Research Station.

For maximal retention of calcium it was necessary to have a daily intake of over 700 mg. of calcium. When a part of the milk calcium of an adequate diet was replaced by an equivalent amount supplied by tri-calcium phosphate in a fortified cereal, utilization as measured by retention was equally good from milk and cereal.

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COMPOSITION OF SOME COMMON FOODS WITH RESPECT TO THE CARBOHYDRATE CONTENT

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In a study in this laboratory on the changes in carbohydrate combustion by a man following ingestion of common foods containing approximately 25 gm. each of carbohydrates, analyses were made of the composition of these foods. In these analyses, supplementing the calculation of the total carbohydrates by difference, determinations were made of the content of reducing sugars, hydrolyzable sugars, and starch. As not all the kinds of food used in this metabolism study (Carpenter, '40) had been previously analyzed in this way and as some of the results differ from the analyses of these foods already published, the values obtained are presented here for purposes of record.

METHODS OF ANALYSIS

In the metabolism experiments, the weight of food to be given to the subject, to contain at least 25 gm. of total carbohydrates, was estimated from the previously published analyses. At the time of each experiment the food was made ready for cooking, if necessary. White potatoes, for example, in amount exceeding the calculated weight, were boiled until soft enough for serving. All visible water was then drained as rapidly and as completely as possible from the potatoes, the portion to be eaten was weighed quickly on a Sauter balance (to 0.1 gm.) and one or two portions were weighed separately as samples for analysis. These samples were dried in an oven at 60°C. until they had lost enough moisture so that they could

415

be ground for analysis. The loss in moisture was determined before grinding, and the water remaining in the sample was also determined by further drying of a small portion of the air-dry sample after it had been ground. The foods were served as promptly as possible after they were cooked and, therefore, contained the maximum amount of moisture. Hence the amounts of carbohydrates in the food portions given to the subject, although estimated from previously published analyses to be 25 gm., were in most instances noticeably lower.

The usual procedures were used for the determinations of nitrogen, fat, and ash. In the calculation of protein, the factor 6.25 was used, to make the results comparable with earlier analyses, although it is recognized that in some instances other factors should be employed. Solutions of glucose and sucrose were prepared and the starch was hydrolyzed in samples of 1 to 3 gm. of each food, according to standard procedures (Association Official Agricultural Chemists, '35). The amounts of the final reducing sugars in the solutions prepared by the several procedures were determined by the method of Folin ('34). To determine the accuracy of the amounts of reducing sugars, hydrolyzable sugars, and starch recovered by the procedures above mentioned, analyses of mixtures of pure glucose, cane sugar, and starch were made.

The cellulose content was determined by the method of Crampton and Maynard ('38) and the heat of combustion by the oxy-calorimeter (Benedict, '29).

<center>RESULTS</center>

The results of the analyses are given in table 1, the order of listing of the foods being the same as will be given later (Carpenter, '40) concerning the metabolism study with these foods. The percentages of the different constituents and the heats of combustion are given on the basis of the fresh food, in the condition as served to the subject.

The rice and the macaroni were characterized by a lack both of reducing and hydrolyzable sugars. The macaroni had a higher percentage of protein than the rice, but the starch con-

TABLE 1

Composition and energy values of foods as served.

FOOD	DATE OF USE	WATER	PRO-TEIN	ETHER EX-TRACT	ASH	Reduc-ing sugars	Hydro-lyzable sugars	Starch	Cellu-lose	UNDE-TER-MINED	HEAT OF COMBUSTION PER GRAM
	1937–1938	%	%	%	%	%	%	%	%	%	*kg.-cal.*
Rice, boiled		75.4	2.0	0.02	0.09	0.0	0.00	23.4	0.60	-1.5	1.00
Macaroni, boiled		71.6	4.5	0.05	0.16	0.0	0.00	23.7	0.34	-0.35	1.22
Bread, white, without crusts	Feb. 18	38.9	9.5	1.7	0.78	3.1	0.40	42.5	1.5	1.6	2.58
	Mar. 16	39.0	9.0	0.98	0.48	3.1	0.26	43.6	1.4	2.2	2.60
	Mar. 21	38.5	9.8	1.7	0.73	2.5	0.00	45.1	2.0	-0.33	2.68
Potatoes, sweet, boiled	Oct. 6	65.3	1.6	0.27	0.75	5.2	5.4	13.8	1.6	6.1	1.34
	Jan. 24	71.6	1.2	0.20	0.66	4.0	6.3	11.4	1.6	3.0	1.12
	Feb. 28	75.6	0.90	0.21	0.60	4.3	2.7	10.8	1.6	3.3	0.96
Potatoes, white, raw	Jan. 28	75.9	1.3	0.11	0.72	0.72	0.67	20.8	0.66	-0.88	0.94
	June 6	79.3	2.0	0.10	0.64	0.00	0.00	18.4	0.79	-1.2	0.82
Potatoes, white, boiled	Oct. 4	81.1	1.8	0.04	0.65	0.29	0.00	16.4		-0.28 [1]	0.75
	Feb. 23	81.8	1.6	0.07	0.48	1.01	0.00	15.0	0.59	-0.55	0.73
Peas (fresh), boiled	May 27	81.7	5.2	0.48	0.50	0.00	2.3	7.0	3.6	-0.78	0.83
	June 1	84.2	4.8	0.62	0.48	0.26	1.0	5.4	2.2	1.04	0.73
Parsnips, boiled	Oct. 13	78.6	1.7	0.54	0.70	1.3	3.1	10.8	1.9	1.4	0.90
	Jan. 17	85.9	1.6	0.72	0.51	1.1	3.4	2.6	2.2	2.0	0.59
	Mar. 4	90.1	1.6	0.60	0.56	1.0	1.1	1.3	1.8	1.9	0.44
Beets, boiled	Dec. 20	92.2	1.2	0.21		0.36	2.8	0.83		2.4 [2]	0.30
	Jan. 12	92.2	1.2	0.18	0.48	0.43	2.3	0.99		2.2 [1]	0.31
	Feb. 25	91.1	1.4	0.17	0.49	0.40	3.6	0.90	1.4	0.54	0.35
Carrots, raw	Jan. 26	91.5	0.66	0.40	0.52	1.4	2.4	0.98	1.5	0.64	0.35
	Jan. 31	90.7	0.79	0.40	0.52	0.93	2.5	1.1	1.7	1.4	0.37
	Mar. 7	90.9	0.67	0.19	0.49	1.8	3.0	1.2	1.4	0.35	0.37
Carrots, boiled	Oct. 8	92.2	0.79	0.22	0.43	1.0	2.0	1.3		2.1 [1]	0.32
	Jan. 10	91.8	0.69	0.40	0.42	1.2	2.0	1.3	1.6	0.59	0.33
	June 3	89.9	0.72	0.60	0.51	1.5	3.1	1.4	1.6	0.67	0.41
Squash, boiled	Dec. 22	89.2	1.7	1.2	0.44	2.6	0.96	2.3	1.6	0.0	0.53
	Jan. 21	95.2	0.34	0.18	0.25	1.4	0.07	0.50		2.1 [1]	0.21
	Mar. 2	91.8	0.96	0.58	0.41	1.7	0.18	0.90	2.0	1.4	0.34
Cashew nuts, raw, unsalted	Apr. 20	3.75	19.9	47.6	2.4	0.0	6.0	13.9	1.7	4.8	6.82
cooked, salted	Mar. 23	3.47	18.1	49.9	2.5	0.0	5.9	20.0	1.3	-1.2	6.94
cooked in cocoanut oil	June 17	1.81	20.0	50.7	2.5	0.0	7.3	15.2	2.3	0.19	7.10
Peanuts, roasted, buttered salted	Mar. 31	3.15	26.5	51.8	2.3	0.0	4.3	12.1	4.0	-4.2	7.05
Almonds, blanched	Apr. 6	3.82	21.9	55.9	3.0	0.0	5.8	8.5		1.1 [1]	7.25
Pecans, raw, unsalted	Apr. 8	2.62	11.0	69.7	1.6	0.0	2.5	7.9		4.7 [1]	8.21
roasted, buttered, salted	Mar. 25	2.25	10.4	72.3	1.6	0.0	3.3	6.0	3.1	1.1	8.20
Walnuts, California, raw	Mar. 28	3.19	17.8	66.5	1.8	0.0	2.1	5.3	4.6	-1.3	7.90
Filberts, raw, unsalted	Apr. 4	3.38	14.9	65.6	2.3	0.0	3.4	5.9	4.7	-0.18	7.91
roasted, unsalted	Apr. 15	1.55	14.1	66.6	2.3	0.0	5.8	6.8	3.9	-1.1	7.99
Dates, dried, pitted		19.6	1.8	0.35	1.4	67.4	0.0	3.1	3.1	3.3	2.92
Figs, pressed, Smyrna	Apr. 27	27.3	3.1	0.34	1.8	45.7	7.9	2.9	6.5	4.5	2.73

[1] Including cellulose.
[2] Including cellulose and ash.

tent of both was practically the same. The white bread contained significant amounts of reducing sugars and some hydrolyzable sugars, but mainly starch; the protein content averaged over 9%.

The boiled sweet potatoes and all the other cooked vegetables were characterized by a high water content. The lowest water content was found with one of the samples of sweet potato and the highest with one of the samples of squash. The sweet potatoes contained a significant amount of reducing sugars, varying amounts of hydrolyzable sugars, and from 11 to 14% starch.

The raw white potatoes, in addition to water, contained for the most part starch. In one sample there were no sugars of any kind and in the other sample a total of only 1.4%. The boiled white potatoes had a somewhat lower content of starch and a slightly higher content of water than did the raw white potatoes. The boiled fresh peas were characterized by a higher protein content than any of the preceding foods except the white bread, and had some hydrolyzable sugars and a significant amount of starch.

In the boiled parsnips there were appreciable amounts of the three classes of available carbohydrates, although these varied considerably, particularly the starch content. This variability in starch content was apparently related to the amount of water present, as the sample with the largest amount of starch had the lowest water content. The boiled beets likewise had noticeable amounts of reducing and hydrolyzable sugars, particularly the latter, and just under 1% starch.

The raw carrots all had significant amounts of the three classes of available carbohydrates, the content of hydrolyzable sugars being the highest, that of reducing sugars next, and that of starch the lowest. The composition of the boiled carrots was not materially different from that of the raw carrots except for the fat content, which averaged a little higher in the former. The boiled squash was one of the most variable of the vegetables in composition, as the water content of the

three samples ranged from 89 to 95% and there was a wide variation in the protein and the fat content.

The nuts were characterized by a low water and a high protein content, the latter ranging from 10.4% in the roasted, buttered pecans to 26.5% in the roasted peanuts. The fat content also was high, as it varied from 47.6% with the raw cashew nuts to 72.3% with the roasted pecans. There were no reducing sugars in any of the nuts, but hydrolyzable sugars were found, ranging from 2.1 to 7.3%. The starch content ranged from 5.3% with raw California walnuts to 20.0% with cooked, salted cashew nuts.

The dried figs and dates were characterized by high amounts of reducing sugars. No hydrolyzable sugars were found in the dates, but there was 8% in the figs. Slight amounts of starch were found in both fruits.

On the fresh basis, the cellulose content was lowest with boiled macaroni and highest with one of the samples of peas, some of the nuts, and the two fruits. The nuts, however, had relatively small amounts of moisture. When the values are calculated to a water-free basis, the differences in the amounts of this constituent are more evident. The rice, macaroni, bread, white potato, and most of the nuts have under 5% cellulose on the water-free basis, the vegetables other than sweet and white potatoes contain from 9 to 24% (the majority over 15%), and the dates and figs have 4 and 9%, respectively.

The amount of undetermined material on the fresh basis was low for the most part, the highest value being 6.1% with sweet potato. In a number of instances the sum of the determined constituents exceeded 100%, which indicates a summation of errors in the several determinations. In these instances the percentages of undetermined material have been given minus signs in table 1. On the water-free basis, the highest values are with boiled sweet potatoes (11 to 18%), boiled parsnips (7 to 20%), raw carrots (4 to 15%), boiled carrots (7 to 8%), and the third sample of squash (16%). Most of the values for the other foods are under 5%. In the vegetables some material was left undetermined by the conventional methods. Lignin was not determined.

The heats of combustion of the various foods per gram of fresh material varied widely. The cooked vegetables had the lowest values. The boiled beets, raw carrots, two of the samples of boiled carrots, and two of the samples of squash had energy contents of under; 400 gm.-calories. For the boiled peas and the white potatoes, both cooked and raw, the values approach nearly 1000 gm.-calories. Of the boiled vegetables, the sweet potatoes had the highest energy content. The nuts, because of their high protein and fat content and low water content, had high energy values, ranging from 6.82 kg.-calories with raw cashew nuts to 8.21 kg.-calories with raw pecans.

When the undetermined material is assumed to have a caloric value of 4 kg.-calories per gram (fresh basis) and the calories in the undetermined material thus calculated are added to the calories calculated for the other known constituents, the agreement between the calculated total caloric value of the food and the caloric value as determined by the oxy-calorimeter is, for the most part, good. The greatest discrepancy is 10%, noted with the boiled squash (see "January 21" in table 1). Most of the other differences range under 5%.

COMPARISON WITH PREVIOUS ANALYSES

In the calculation of the amount of food to be given to the subject to supply 25 gm. of carbohydrates, several sources of data were consulted as a theoretical basis. [1] These were the compilation of analytical results made by the United States Department of Agriculture (Atwater and Bryant, '02), and the reports of McLester ('28), McCance, Widdowson and Shackleton ('36), and Mitchell and Beadles ('37). To compare the results in table 1 with these previous analyses, the water

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[1] Olmsted and Williams ('39) have reported that by their method of analysis they have been able to account for more than 98% of the carbohydrate in a variety of common foods, but they give no tabulated results.

content in the several foods must be known, because the amount of water in the material determines the general level of the other constituents.

The water content was essentially the same as reported in earlier studies in the case of boiled rice, raw white potato, raw and cooked carrots, and all the nuts, lower in boiled macaroni, and higher in the other foods. When the other constituents are calculated on the basis of water-free material, comparison shows that the percentages of total carbohydrates were approximately the same in white bread, raw white potato, peas, raw carrots, the nuts, and the fruits as in these same types of food previously analyzed. The values for total carbohydrates in squash were variable and not comparable with values reported previously. They were higher in rice, macaroni, sweet potato, and beets, and lower in the other foods.

The distribution of carbohydrates in the sweet potatoes, the raw white potatoes, and the green peas was not materially different from earlier findings. The parsnips contained reducing sugars whereas the earlier analyses showed none, but the content of hydrolyzable sugars was about the same in both instances. The beets contained a smaller percentage of hydrolyzable sugars, the raw and cooked carrots a smaller percentage of reducing sugars, and the cooked carrots nearly the same percentage of hydrolyzable sugars as previously reported.

The green peas contained a larger proportion of protein and the parsnips about the same proportion. There was some starch in the raw carrots whereas none was found in the previous analyses. The starch content was greater in the boiled carrots and the squash. The protein and fat content of the nuts and the dried fruits were much the same as reported before.

The chemical analyses of the foods were made by Mr. Martin Stankard and the heats of combustion by Mr. Basil James.

SUMMARY

Twenty kinds of common foods, including rice, macaroni, white bread, vegetables (raw and cooked), nuts, dates, and figs, were analyzed with special reference to their content of reducing sugars, hydrolyzable sugars, starch, and cellulose. Determinations were also made of the water, protein, fat, and ash content, and the heats of combustion. The results obtained are compared with previous analyses of similar foods by other investigators.

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THE COMBUSTION OF CARBOHYDRATES IN MAN AFTER INGESTION OF COMMON FOODS

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Many studies have already been made of the respiratory exchange and the combustion of carbohydrates after ingestion of various types of pure sugars, either singly or in combination. However, the foods commonly eaten by man (table sugar or sucrose, maple sugar, and honey excepted) do not consist of pure sugars alone but contain the more complex carbohydrates and other materials such as protein, fat, ash, and varying amounts of water. Man's main sources of energy from carbohydrates are the sugar in milk and the sugars and starches in fruits, cereals, and vegetables. The rapidity with which carbohydrates in these foods are burned in the body after ingestion and the amounts burned may vary greatly. A special study was therefore made of the combustion of carbohydrates by man after ingestion of various common foods that contain not only the simple sugars but the more complex carbohydrates. The object of this study was to secure information as to how quickly a certain amount of carbohydrates in various foods would be available for combustion in the body and how much carbohydrate would be burned in a given time.

EXPERIMENTAL

The subject was a man (age, 23 years; height, 176 cm.; initial weight, 65.8 kg.; final weight, 70.6 kg.), a medical student, apparently in good health. His respiratory exchange

423

was measured by the open-circuit helmet respiration apparatus (Benedict, '33). After a preliminary 15-minute period of breathing in the helmet, there were three 15-minute base line (sitting, post-absorptive) periods of measurement, followed by an interval during which the subject ate the food (also drinking 250 cc. of water at room temperature), and then by twelve successive 15-minute periods. All experiments were in the morning, usually ending between 1 P.M. and 2 P.M. The subject voided urine upon arrival at the laboratory, about 8 A.M., and again after the experiment ended. The partition of the carbohydrate, fat, and protein combustion in the total metabolism was calculated in the conventional manner, the protein combustion being estimated from the grams of nitrogen (multiplied by the factor for body protein, 6.0) eliminated in the second voiding of urine.

Analyses of the ventilating air current of the helmet apparatus were made with the gas-analysis apparatus of Carpenter ('33). Fifty-nine analyses of outdoor air gave an average of 0.032% for carbon dioxide and 20.940% for oxygen, with standard deviations of 0.0015 and 0.0043 for the two gases, respectively. Fifteen analyses of air from the helmet when alcohol was burned in it gave an average respiratory quotient of 0.670, with a standard deviation of 0.0071.

<div align="center">RESULTS</div>

Foods eaten and amounts to give 25 gm. of available carbohydrates. It was planned to give such an amount of food in edible condition as would contain a total of 25 gm. of available carbohydrates. The amounts of the different foods necessary for this purpose were calculated from previous analyses made by other investigators. At the time of each metabolism experiment, a sample of the particular food served was saved for subsequent analysis. The results of these analyses have been given in a preceding publication (Carpenter, '40). As there pointed out, because of the quickness of serving the foods after they were cooked, the water content was greater

in most instances than found in the previous analyses. Hence the available carbohydrates in the foods as eaten (fifth column, table 1) were in most instances under 25 gm. Subsequently calculations were made (based upon the actual analyses of the foods) of the amounts of the foods that should have been eaten in order for the subject to get 25 gm. of available carbohydrates. These calculated amounts differ greatly. For raw white potato and raw carrots the amounts were somewhat less than those of the same vegetables when cooked, perhaps because soluble carbohydrates leached from the vegetables as they were cooking or because the vegetables took on water while boiling. The amounts of dried dates and figs were but little more than those of cane sugar and glucose.

Increases in carbohydrate combustion during 3 hours after food ingestion. In table 1 are also recorded the carbohydrate combustion of the subject when post-absorptive (base line), the increases in carbohydrate combustion above the base lines as measured during the 3 hours immediately following ingestion of the foods, and the calculated increases that might have occurred in this time, had each food portion contained 25 gm. of available carbohydrates. In this calculation it was assumed that the ratio between the increase in combustion as measured and the theoretical increase after ingestion of 25 gm. of available carbohydrates would be the same as the ratio between the available carbohydrates in the food as eaten and 25 gm. The following discussions deal with the increases as calculated on this basis. In three of the four water experiments the increases in carbohydrate combustion were over 5 gm., indicating that water alone actually increases the combustion. In several of the food experiments the increases were no greater than after water alone and may have been the result wholly of the water drunk when the foods were eaten. No allowance has been made in any of the results for increase caused by water alone, as it is uncertain whether there is a summation of effects when dry food and water are consumed at the same time. With glucose the increases were much the same as with cane sugar. With dates and figs they

were somewhat greater. The uncooked potato starch did not result in as great an increase as the cooked potato starch nor even as great as the water alone. With boiled rice and macaroni, the increases were similar to those with water alone. When raw white potatoes were eaten, the effect on the carbohydrate combustion was not so great as the effect of water alone. Decidedly larger increases occurred after the ingestion of boiled than raw white potato, but a slightly smaller increase, on the average, was found after eating boiled as contrasted with raw carrots. It is generally believed that cooking of vegetables increases their digestibility. The experiments with carrots indicate that there is considerable absorption of the carbohydrate content in the raw form, but those with white potatoes show only a small absorption in this form. The difference between these two raw foods is probably ascribable to the difference in the nature of the carbohydrate content, carrots having more soluble carbohydrates than starch, and white potatoes having predominantly starch. In half of the instances the nuts resulted in smaller increases than did water alone. The boiled vegetables that may be characterized as sweet (parsnips, beets, carrots, and squash) caused the greatest increases. The nuts and the foods rich in starch, like rice, macaroni, white potato, and bread, gave the smallest increases. When dates, cane sugar, and glucose were eaten, the increases were only about two-thirds of those noted after beets and carrots.

Increases in carbohydrate combustion compared with character of food constituents. The increases in carbohydrate combustion were related to some extent to the amounts of reducing sugars in the several foods. When there were no reducing sugars in the foods, the increases per 3 hours were low, except for cane sugar. When the foods contained up to 2 gm. of reducing sugars, the increases were higher. When they contained between 2 to 4 gm., the increases were still higher, but when they contained from 5 to 17 gm., the increases averaged about the same as in the 2- to 4-gm. group. The foods having from 20 to 25 gm. of reducing sugars, on the

contrary, caused increases that were not much greater than those which occurred with the foods having only up to 2 gm. The increases were, in general, smaller with the foods that contained no hydrolyzable sugars, although the relationship was not marked. The smaller the starch content of the food, the greater was the increase. The carbohydrate combustion was more dependent upon the presence of reducing and hydrolyzable sugars than upon the presence of starch in the foods. Because of the great difference between the fat content of the nuts and the other foods, it is difficult to make a comparison with this food constituent, except to state that the increases in carbohydrate combustion were generally lower with the foods (nuts) having the very high fat content.

Course of carbohydrate combustion after food ingestion. In figures 1 to 5 are indicated the average level of carbohydrate combustion per 15 minutes in the three base line periods and the average levels in the successive 15-minute periods following ingestion of each type of food studied. Each curve represents the average of the results obtained in the several experiments with the particular food (except as indicated by the footnote in table 1), corrected to the basis of a food portion containing 25 gm. of available carbohydrates. The base line carbohydrate combustion in the individual experiments varied from 0.41 to 1.75 gm. per 15 minutes and averaged 1.12 gm. The base line respiratory quotient ranged from 0.75 to 0.85 and averaged 0.80. The changes in respiratory quotient in the successive 15-minute periods after food ingestion closely paralleled the changes in carbohydrate combustion.

There was a marked difference between the availability of glucose and of cane sugar. With cane sugar (fig. 1) there was an immediate maximum rise in carbohydrate combustion in the second quarter hour after ingestion (greater than that noted with any of the other foods), followed by a sharp decrease, an approach to the base line after the first hour, and a return to it at the end of 3 hours. The rise after glucose (fig. 4) was much less marked during the first three 15-minute

periods, and the maximum rise did not occur until the sixth period. Thereafter the carbohydrate combustion decreased gradually, but even at the end of 3 hours it was still appreciably above the base line. Apparently glucose is used first to fill the glycogen reserves of the body, after which it becomes available for combustion, and the combustion lasts a longer time than with cane sugar.

The course of the carbohydrate combustion after ingestion of dates (fig. 1) closely resembled that after cane sugar, the maximum increase occurring in the same period, although it was not so high as after cane sugar and the combustion did not return to the pre-ingestion level at the end of 3 hours. With figs (fig. 2) the maximum increase appeared later than with dates and was not so great, but the subsequent increases were greater.

It has been shown in earlier studies (Carpenter, Bensley, Dill and Edwards, '37) that the initial rise in the respiratory quotient after ingestion of fructose is caused by the formation of lactic acid. It is probable that the early maximum rise in carbohydrate combustion noted both with cane sugar and with dates is also ascribable to the fructose in these two materials.

After rice (fig. 1) there were small increases during the first three periods, the maximum was not reached until the beginning of the third hour, and there was no return to the base line at the end of 3 hours. In one experiment, continued for 4 hours, the combustion was still above the base line at the end of that time. Rice, therefore, is a food whose carbohydrate content is only slowly available to the body, and a long time is required for its combustion.

With beets the increase was immediate, but the maximum did not occur until the sixth period, and the combustion was still at a high level at the end of 3 hours. After white bread (fig. 2) the maximum rise occurred in the fifth quarter hour, following which the combustion gradually decreased, but at the end of 3 hours the base line was not reached. There was a distinct difference between the effects of boiled and raw white potatoes, the maximum rise with raw potatoes being

CARBOHYDRATE COMBUSTION AFTER INGESTION OF 25 GRAMS OF AVAILABLE CARBOHYDRATES IN FOODS

Figure 1

CARBOHYDRATE COMBUSTION AFTER INGESTION OF 25 GRAMS OF AVAILABLE CARBOHYDRATES IN FOODS
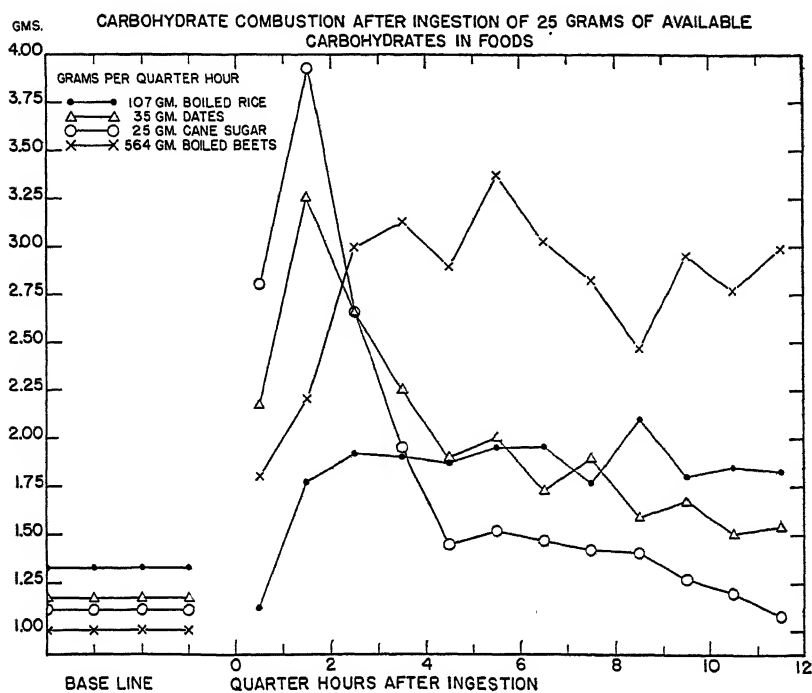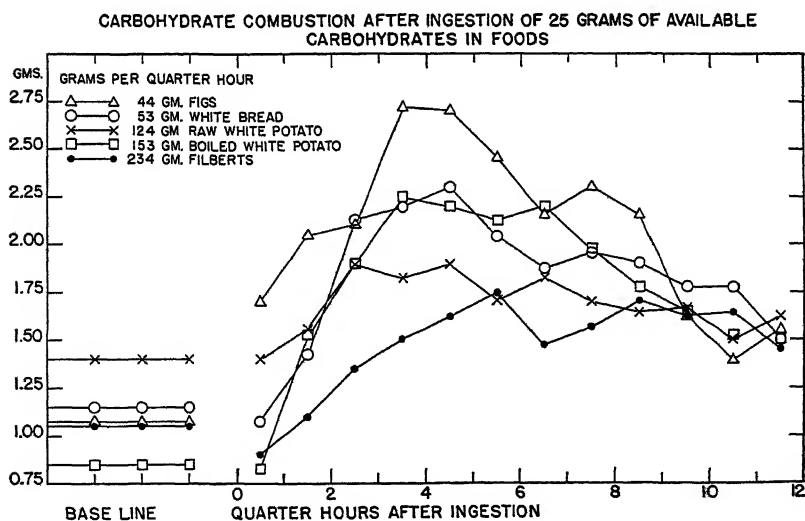
Figure 2

about one-third that with boiled potatoes, although there was not much difference with respect to the time when the maximum rises were found. In the last hour the carbohydrate combustion after ingestion of raw potatoes closely approached the initial level whereas after boiled white potatoes it was still considerably above the initial level.

The increases in carbohydrate combustion were greater in the first hour and the maximum rise occurred sooner after raw than after boiled carrots (fig. 3). In the last hour the results were essentially the same in both instances.

The parsnips (fig. 4) were slowly digested, continually furnishing significant amounts of carbohydrates. The maximum increase in carbohydrate combustion did not occur until the third hour, although a marked rise was found in the first hour. Apparently the readily digestible carbohydrates were burned first, and subsequently more carbohydrates were liberated and made available. With boiled sweet potatoes and with peas (fig. 5) the increase in combustion was nearly over in 3 hours. However, with squash there was no indication of a return to the initial level after 3 hours. With macaroni, although the maximum occurred in the last two periods, there was no great change in the combustion after the fourth period. At the end of 3 hours the combustion was still above the initial level.

The nuts gave varying results, although in the majority of cases the maximum increases appeared early, from the third to the seventh quarter hour. In one instance (almonds) the maximum increase came in the twelfth period. However, the maximum increases with the nuts were small and, on the average, not above 0.85 gm. in any case. When once the maximum increase for any particular nut was attained, the combustion continued at about the same level throughout the remainder of the 3 hours. The increases were somewhat greater after cashew nuts than after the other nuts, although the increases after peanuts were almost as great.

In general, the foods that contained readily available amounts of reducing sugars or hydrolyzable sugars resulted in
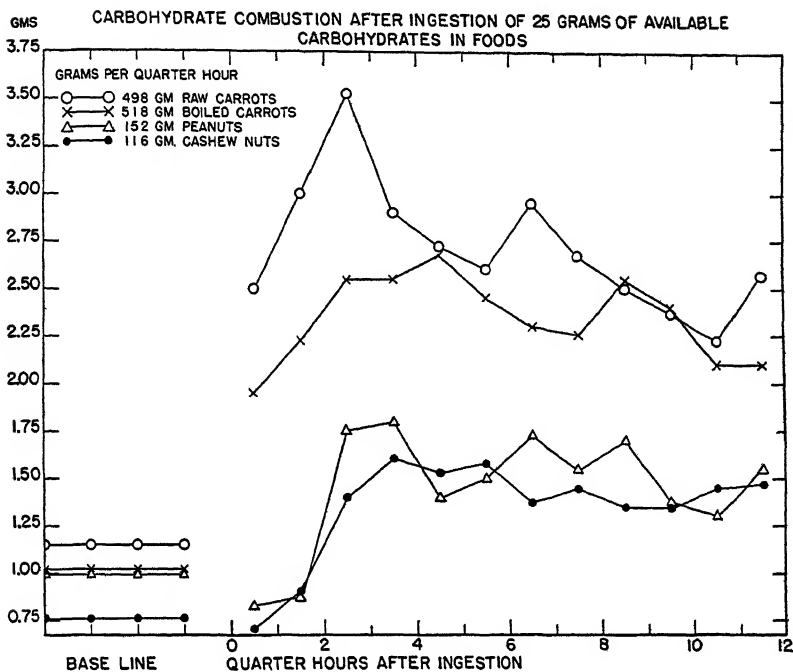
Figure 3
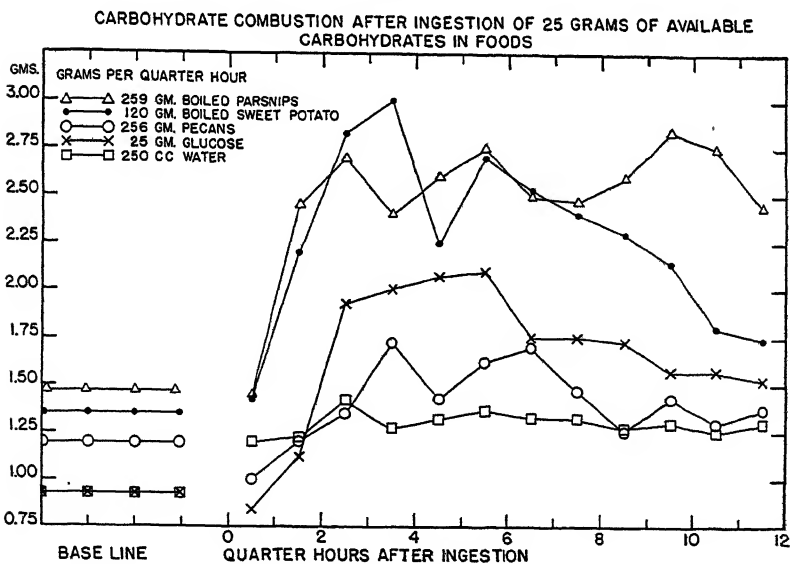
Figure 4

greater and earlier maximum rises in carbohydrate combustion per 15 minutes than did the foods with high starch content and those (nuts) with high fat content, but the increased combustion lasted a shorter time.

The measurements of the respiratory exchange were made by Mr. Basil James.

CARBOHYDRATE COMBUSTION AFTER INGESTION OF 25 GRAMS OF AVAILABLE CARBOHYDRATES IN FOODS

GRAMS PER QUARTER HOUR
- ● 336 GM. WALNUTS
- ○ 119 GM. BOILED MACARONI
- □ 643 GM. BOILED SQUASH
- × 320 GM. BOILED PEAS
- △ 174 GM. ALMONDS

BASE LINE   QUARTER HOURS AFTER INGESTION
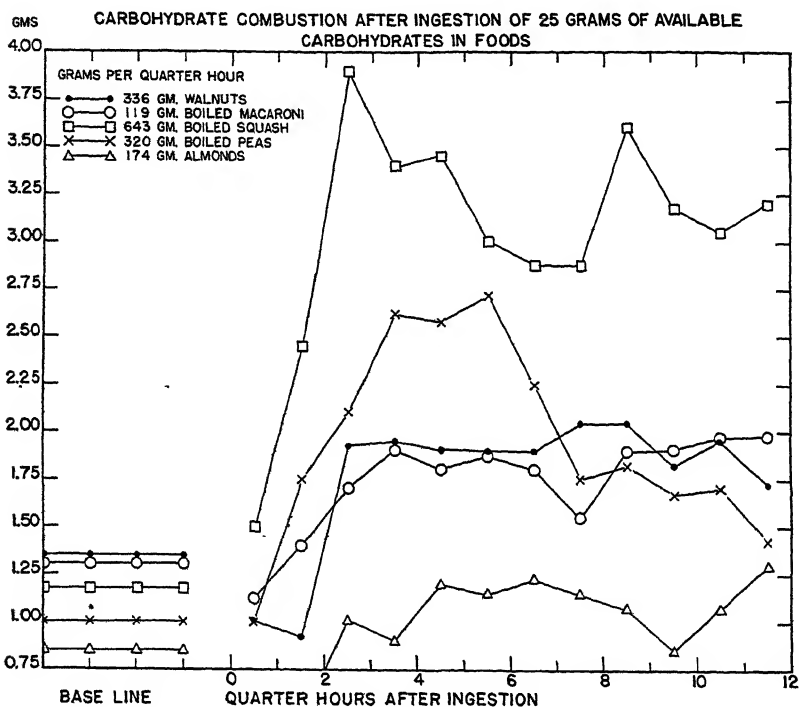
Figure 5

SUMMARY

From measurements of total respiratory exchange and urinary nitrogen elimination of a man before and after ingestion of portions of common foods, each containing approximately 25 gm. of available carbohydrates, calculations were made of the carbohydrate combustion in the post-absorptive condition and in twelve successive 15-minute periods immediately after food ingestion. The amounts required

of the several foods to give 25 gm. of available carbohydrates differed greatly. The increases in carbohydrate combustion during the 3 hours following food ingestion were greater, the greater the amounts of reducing and hydrolyzable sugars in the foods, and smaller the greater the amounts of starch or fat in the foods. The boiled vegetables that may be characterized as sweet (parsnips, beets, carrots and squash) caused the greatest increases. Nuts, rice, macaroni, white potato, and bread caused the smallest increases. The combustion of carbohydrates was greater when carrots were eaten raw than when eaten cooked, but the picture was the reverse with white potatoes. The increase in combustion was sudden and marked but quickly over with cane sugar and dates. That with glucose was slower and less marked but lasted longer. With parsnips the readily digestible carbohydrates were burned first; subsequently the more complex carbohydrates were liberated and made available. With nuts the increases in carbohydrate combustion were small but continuous; they were somewhat greater with cashew nuts than with other nuts.

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CHOLINE METABOLISM

IV. THE RELATION OF THE AGE, WEIGHT AND SEX OF YOUNG RATS
TO THE OCCURRENCE OF HEMORRHAGIC DEGENERATION
ON A LOW CHOLINE DIET

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The lack of an adequate amount of choline in the diet of young rats has been found to produce a severely toxic condition to. which the name hemorrhagic degeneration has been applied. This deficiency was characterized by the rapid onset of acute renal hemorrhage which could be prevented by the inclusion in the diet of less than one-half the quantity of choline required to avert the formation of a fatty liver. The need for choline was shown to be greater on diets containing added cystine, protein rich in cystine, cholesterol or fat. Methionine exhibited a choline-like action opposite to that of cystine. All of these results were obtained in male rats, 38 to 42 gm. in weight and 22 to 26 days of age (Griffith and Wade, '39 and '40; Griffith, '40). Inasmuch as certain of the experiments suggested a relationship of age or weight to the severity of the ensuing pathological state, a study has been made of the effect of these factors and of sex on the development of this recently recognized result of choline deficiency.

EXPERIMENTAL

The experimental procedures and the constituents of the diet were those previously described (Griffith and Wade, '39). Determinations were made of liver and kidney weights and of

the total chloroform-soluble substances in the liver. The term "liver fat" in tables 1 to 5 refers to this fraction.

The basal diet, AC34, consisted of casein 15%, lard 9, sucrose 62.9, salt mixture 4 (Hawk and Oser, '31), calcium carbonate 1, agar 2, whole dried yeast 6, and the fortified fish liver oil, Natola,[1] 0.1%, respectively. Diets AC32 and AC33 contained 0.3 and 1.0% of added cystine respectively.

The appearance of the kidneys is recorded in tables 1 to 4 as hemorrhagic, partially hemorrhagic, recovered, or normal. The renal hemorrhagic degeneration observed in 40-gm. male rats on diet AC32 resulted in the transformation on the fifth and sixth days of an apparently normal kidney into a greatly enlarged dark red, bloody structure. The mortality was highest on the seventh day. If the animal survived this acute stage the recovery phase was almost as rapid as the degenerative phase. The dark red color of the cortex was replaced by a mottled or blotched light brown appearance, quite distinct from that of the normal kidney. If the hemorrhage was not severe the kidneys were nearly normal, except in size, on the tenth day. In many rats, presumably those more severely affected, the "recovered" kidneys had a frosted appearance due to a white incrustation on the cortex. Kidneys were recorded as normal, if no trace of hemorrhage or of the recovery appearance was evident; as hemorrhagic, if the renal lesion, whether moderate or severe, showed no sign of the recovery process; as partially recovered, if the cortex showed areas of hemorrhage and areas of recovery; and, as recovered, if the cortex showed the mottled surface without any remaining hemorrhagic areas.

<center>RESULTS</center>

Relation of age of 40-gm. male rats to hemorrhagic degeneration. Table 1 shows the relation of the age of the young male rats placed on the choline deficient diet to the severity and duration of the acute phase of this deficiency. The initial

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[1] We wish to thank Parke, Davis and Co. for a generous supply of Natola.

weight of these rats was essentially the same, 38 to 42 gm., so that group 1 represented the most rapidly growing and group 8 the least rapidly growing young of this rat colony. These rats were weaned on the twentieth day so that group 1 was placed immediately on the experimental diet and group 8 remained on the stock diet for 7 days before reaching the 40 gm.

TABLE 1

The relation of age to deposition of liver fat and to occurrence of hemorrhagic degeneration in 40-gm. male rats during a 10-day experimental period on a low choline diet

GROUP NO. AND DIET	1 AC32	2 AC32	3 AC32	4 AO32	5 AC32	6 AC32	7 AC32	8 AC32	9 AO33
Age, days	20	21	22	23	24	25	26	27	22
No. of rats in group	43	41	44	44	46	52	40	42	18
Mortality, %	5	24	33	43	37	25	20	17	6
Average final body weight, gm.	57	54	54	50	56	57	57	60	51
Average weight of liver, gm.	4.21	3.96	3.88	3.49	4.06	4.35	4.33	4.64	4.12
Average weight of liver fat, mg.	1140	952	988	745	928	1083	1115	1105	865
Average weight of kidneys (per pair), mg.	924	910	860	892	850	868	882	900	1044
Kidney weight as per cent of normal kidney weight	138	142	134	146	129	130	132	130	171
Appearance of kidneys Normal, %	12	10	14	2	9	12	17	19	6
Recovered, %	26	20	16	14	15	29	8	19	22
Partially recovered, %	58	37	25	39	37	25	50	29	33
Hemorrhagic,[1] %	4	33	45	45	39	34	25	33	39

[1] Figures include rats which died during the experimental period.

starting weight. Over 80% of the rats in each group showed evidence of renal pathology on the tenth day. It was probable that most of the remaining 20% were actually "recovered" animals because autopsy of similar rats on the sixth and seventh days showed that 95% or more were moderately or

severely hemorrhagic. Although there was no significant dif- ·
ference in the incidence of the deficiency in these eight age
groups, it was evident that the rats in group 1 were less
severely affected than the others. Fewer of these rats died
during the experimental period and on the tenth day more
were in the recovery phase than was true for any of the other
groups. The possibility that this difference may have been due
to the protective milk diet of the immediately preceding lacta-
tion period is being investigated.

The highest mortality, 43%, occurred in rats 23 days of age
(group 4). Mortality increased as the starting age of the ani-
mals increased from 20 to 23 days of age and then decreased
in the older animals. Group 9 shows the effect of increasing
the cystine content of the diet from 0.3% (AC32) to 1%
(AC33). The hemorrhagic enlargement of the kidneys was
intensified, although the mortality was less than that found in
the same age group fed diet AC32 (group 3). The usual fatty
livers occurred in all of the rats in table 1. Hemorrhage into
the eyeball occurred in 20% of these animals.

*Relation of age and weight of male rats to hemorrhagic de-
generation.* The results in table 2 show the occurrence of
hemorrhagic degeneration in normal male rats, 24 to 41 days
of age, on diets AC32 and AC33. Rats, 65 gm. in weight and
30 days of age, showed the same incidence of the deficiency as
the 40-gm. rats reported in table 1. However, the acute stage
was not reached until the tenth day (group 3) instead of the
sixth day as in the case of the 40-gm. rats (group 1). Recovery
occurred in most of these rats by the fifteenth day (group 4).

The effects of choline deficiency were decidedly less evident
in rats over 30 days of age (groups 5 to 9). Only 25 to 30%
of these older animals showed renal lesions. If renal degenera-
tion occurred, it was moderate rather than severe. None of
the animals died. The 10-day period was not too short a period
for the production of the deficiency in the older animals be-
cause these rats showed fewer signs of renal pathology after
15 days (group 6) than after 10 days (group 5). The use of
diet AC33 with its higher concentration of added cystine did

not alter the results (groups 8 and 9). This difference between 30- and 33-day-old rats was unusually interesting because it supported a similar observation noted in the first paper of this series (Griffith and Wade, '39). At that time it was found that the deposition of liver fat in rats over 35 days of age was only

TABLE 2

The relation of age and weight to deposition of liver fat and to occurrence of hemorrhagic degeneration in male rats on a low choline diet

GROUP NO. AND DIET	1 AC32	2 AC32	3 AC32	4 AC32	5 AC32	6 AC32	7 AC32	8 AC33	9 AC33
Age, days	24	30	30	30	33	33	37	36	41
Experimental period, days	6	6	10	15	10	15	10	10	10
No. of rats in group	29	14	20	21	15	16	12	19	21
Average initial body weight, gm.	40	63	65	66	72	71	82	73	88
Average final body weight, gm.	54	83	88	111	107	122	112	103	124
Average weight of liver, gm.	3.76	5.04	5.76	8.33	7.39	9.78	7.53	6.95	8.68
Average weight of liver fat, mg.	988	1060	1572	2275	1968	....	1445	1565	1765
Average weight of kidneys (per pair), mg.	795	797	1288	1217	1050	1202	1155	1082	1385
Appearance of kidneys Normal, %	7	93	10	38	73	94	75	63	62
Recovered, %	0	0	0	43	0	6	0	5	10
Partially recovered, %	0	0	15	0	0	0	0	0	0
Hemorrhagic, %	93	7	75	19	27	0	25	32	38

one-half that occurring in rats less than 30 days of age. Apparently there is a marked reduction in the choline requirement of young male rats after reaching 30 days of age.

This relation of age to the occurrence and severity of hemorrhagic degeneration in young male rats is also shown in figure 1. These curves were drawn from data which confirmed in every respect the results noted in tables 1 and 2 on smaller groups of animals. Curve I shows the high incidence of renal

lesions in rats 30 days of age or less and the sharp drop in incidence in rats 33 days of age or older. Curve II shows the per cent of rats with severe renal hemorrhage at the end of the 10-day period. At this time rats under 27 days of age were largely in the recovery phase whereas most of the rats, 30 days of age, were in the acute stage. Curve III shows the per cent of rats which had partially or wholly recovered from the hemorrhagic state. Such recovery was particularly evident in rats 20 days of age. Curve IV shows the mortality in the various age groups. Mortality was highest in rats 23 days of age.
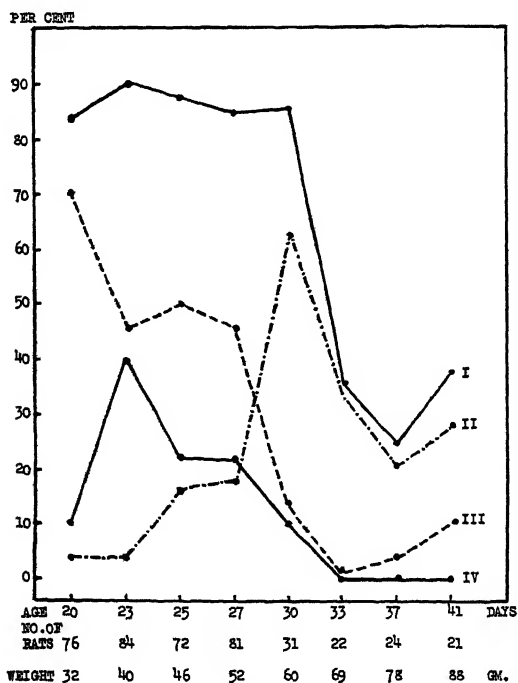
Fig. 1   The relation of age and weight to the occurrence and severity of renal hemorrhagic degeneration in young male rats on a low choline diet (AC32).

Curve I. Per cent of rats showing renal lesions during the 10-day period.

Curve II. Per cent of rats showing hemorrhagic lesions at the end of the 10-day period.

Curve III. Per cent of rats showing partial or complete disappearance of hemorrhagic lesions at the end of the 10-day period.

Curve IV. Per cent mortality during the 10-day period.

None of the rats, 33 days of age or older, failed to survive the 10-day experimental period.

Incidence of hemorrhagic degeneration in 40-gm. female rats. The results of choline deficiency were less severe in female rats than in male rats of the same age and weight. A longer experimental period was required for the production of the deficiency, and the degenerative enlargement of the kidneys

TABLE 3

The deposition of liver fat and the occurrence of hemorrhagic degeneration in 40-gm. female rats on a low choline diet

GROUP NO. AND DIET	1 AC32	2 AC32	3 AC32	4 AC33	5 AC33
Experimental period, days	6	10	15	6	10
No. of rats in group	15	22	15	10	21
Average final body weight, gm.	47	61	65	51	63
Average weight of liver, gm.	2.98	4.86	5.23	3.82	5.21
Average weight of liver fat, mg.	635	1288	1271	935	1690
Per cent of liver fat in liver	21.3	26.4	24.3	24.4	32.4
Average weight of kidneys (per pair), mg.	538	780	775	606	850
Kidney weight as per cent of normal kidney weight	95	112	107	99	119
Appearance of kidneys					
Normal, %	100	23	40	100	19
Recovered, %	0	23	46	0	14
Partially recovered, %	0	14	7	0	9
Hemorrhagic, %	0	38	7	0	58

was not as marked as in males. The results of these experiments on female rats, 38 to 42 gm. in weight and 24 to 26 days of age, are shown in table 3. There was no indication whatever of renal hemorrhage after a 6-day period on diet AC32 (group 1) or on diet AC33 (group 4). Severe hemorrhage occurred in twenty-seven out of twenty-nine young males after a 6-day period on diet AC32 (group 1, table 2). The characteristic results of the deficiency were observed after a 10-day period on these two diets (groups 2 and 5). All of the rats survived and recovery was nearly complete after a 15-day

period (group 3). The usual deposition of liver fat occurred in these animals.

Curative action of choline. The effect of the administration of choline after the appearance of renal lesions is shown in table 4. Four groups of male rats, 38 to 42 gm. in weight and 23 to 24 days of age were fed diet AC32. Groups 1, 2 and 3 (table 4) were killed after 5, 6, and 10 days respectively. On the sixth day the rats in group 4 were changed to diet AC32

TABLE 4

The effect of administration of choline after appearance of hemorrhagic degeneration in 40-gm. male rats on diet AC32

GROUP NO.	1	2	3	4 [1]
Experimental period, days	5	6	10	10
No. of rats in group	30	29	30	33
Average final body weight, gm.	50	54	53	58
Average weight of liver, gm.	3.64	3.76	3.80	3.68
Average weight of liver fat, mg.	942	988	858	552
Average weight of kidneys (per pair), mg.	630	795	870	788
Kidney weight as per cent of normal kidney weight	105	124	140	117
Appearance of kidneys				
Normal, no. of rats	23	2	2	10
Recovered, no. of rats	0	0	5	17
Partially recovered, no. of rats	0	0	11	5
Hemorrhagic, no. of rats	7	27	1	0
No. of deaths	0	0	11	1

[1] Diet AC32 for 6 days. From sixth to tenth days diet AC32 supplemented with 3.0 mg. of choline chloride per gram of food. Two subcutaneous injections of 5 mg. of choline chloride on both sixth and seventh days.

supplemented with 3.0 mg. of choline chloride per gram of food. In addition each rat was given two subcutaneous injections of a solution of choline chloride (5 mg. per 0.25 cc.) on both the sixth and seventh days. Groups 1 and 2 illustrate the very rapid onset of the hemorrhagic condition. Only two out of twenty-nine rats failed to show the renal lesions on the sixth day. After 10 days eleven rats had succumbed to the toxic effects of the choline deficiency, the majority of the fatalities

having occurred on the seventh day (group 3). On the tenth day the hemorrhagic condition was present in twelve of the nineteen surviving rats in group 3 but in eleven of the twelve rats the recovery process had started as evidenced by the partial disappearance of blood from small areas on the cortex. Even though the recovery of surviving rats was rapid without added choline the administration of choline greatly accelerated the process. On the tenth day the rats in group 4, which had been given choline beginning with the sixth day, were definitely in better condition than the rats in group 3. Only one rat failed to respond to the administration of choline. This animal was nearly moribund on the sixth day and died on the seventh day. Of the thirty-two survivors only five showed remaining hemorrhagic areas and in every case these areas were small. This curative action of choline was also illustrated by the changes in the weight of the kidneys. It was assumed that this weight was approximately the same in groups 2, 3 and 4 on the sixth day. The weight continued to increase in the untreated rats (group 3) and decreased in the treated animals (group 4). The 4 days of choline administration resulted in the removal of some but not all of the extra fat deposited in the liver during the first 6 days.

The injection of choline during the acute stage of the renal degeneration was necessary in order to demonstrate its beneficial effect. It was found in preliminary experiments that the consumption of food on the sixth and seventh days, at which time the rats were acutely ill, was too low to supply sufficient choline if only 3 mg. were added per gram of food.

Protective action of choline. The observations on the relation of age and weight to the occurrence and severity of hemorrhagic degeneration indicated that young male rats, 20 days of age, were the most satisfactory animals for the demonstration of the result of choline deficiency and of the protective effect of choline and of choline-like substances. In such rats the incidence of the deficiency was high but the mortality was low. Furthermore, it was evident that more consistent results could be obtained by using a 7-day rather than a 10-day ex-

perimental period. The shorter period permitted the determination of the renal damage during the acute phase uncomplicated by partial recovery of the surviving rats. Table 5 shows the results obtained with male rats, 20 days of age and 24 to 30 gm. in weight, after a 7-day period on the experimental

TABLE 5

The effect of choline on occurrence and severity of hemorrhagic degeneration in 20-day-old male rats, during a 7-day experimental period

GROUP NO. AND DIET	1 AC34	2 AC34	3 AC34	4 AC34	5 AC32	6 AC32	7 AC32	8 AC32	9 AC32	10 AC32	11 AC32
No. of rats in group	21	11	19	13	30	11	10	11	10	10	10
Average initial body weight, gm.	28	26	27	27	28	28	26	27	27	28	28
Average final body weight, gm.	39	40	42	44	34	41	40	40	48	47	45
Choline chloride added per gm. of food, mg.	0	0.06	0.12	0.25	0	0.06	0.12	0.25	0.50	0.75	1.00
Average weight of liver, gm.	2.69	2.89	2.90	2.90	2.31	2.78	2.83	2.64	3.05	2.67	2.15
Liver weight as per cent of body weight [1]	6.83	7.24	6.95	6.64	6.80	6.78	7.07	6.60	6.35	5.68	4.77
Average weight of kidneys (per pair), mg.	678	591	544	517	709	772	707	577	526	533	505
Kidney weight as per cent of body weight [2]	1.72	1.48	1.31	1.16	2.08	1.88	1.76	1.49	1.10	1.13	1.12
Appearance of kidneys Normal, %	10	36	68	100	7	0	0	9	100	100	100
Moderately hemorrhagic, %	23	46	27	0	13	18	40	45	0	0	0
Severely hemorrhagic, %	67	18	5	0	80	82	60	46	0	0	0

[1] Normal per cent of body weight for rats in this colony, 4.31.
[2] Normal per cent of body weight for rats in this colony, 1.1 to 1.2.

diet. The two diets used, AC34 and AC32, were identical except for the addition of 0.3% of cystine to the latter. Although the weights of the livers were included in the results shown in table 5, this measure of the fatty liver effect appeared of far less value in estimating the severity of the deficiency than the weight and appearance of the kidneys. The latter were found to be exceedingly sensitive indicators of the absence of

choline in the diet not only because renal hemorrhage occurred as a sign of the deficiency but also because the resulting renal enlargement varied with the extent of the deficiency. The presence of even slightly hemorrhagic areas in the cortex was readily recognizable by direct observation.

The weight of the kidneys of young normal rats was 1.0 to 1.2% of the body weight. After the onset of hemorrhagic degeneration this value was increased to 1.72% on the basal diet (group 1) and to 2.08% on the basal diet plus 0.3% of cystine (group 5). Groups 2 to 4 and 6 to 11 show the correlation between the weight and appearance of the kidneys and the addition of choline to these two diets. These results indicated that, in the range of suboptimal levels of choline, there was almost a straight line relationship between the choline supplement and the kidney weight, expressed as per cent of body weight. A study is being made of the possibility of using this response of the kidneys to a lack of choline for the assay of this and related substances having a choline-like action or an opposite effect. This latter effect is illustrated in table 5 in the comparison of the protective levels of choline on the basal diet with and without added cystine (groups 4 and 9).

The severity of the renal pathology observed in this investigation emphasized the necessity of additions of choline to such experimental diets. The food consumption of the rats on diet AC34 was 3 to 4 gm. per day so that approximately 1 mg. of choline chloride was required daily to prevent the renal lesions (group 4, table 5). Twice as much was necessary if 0.3% of cystine was included in the ration (group 9). Previous experiments have indicated that the choline requirement for the prevention of fatty livers is two to three times that for the prevention of hemorrhagic degeneration.

Diet AC34 was similar to many which have been used in recent years in investigations in which the young rat has been the experimental animal. It is possible that the deficiency has occurred previously and escaped detection because the rats did not happen to have been sacrificed during the acute stage. Rats which have survived a moderately hemorrhagic condition show normal early growth curves except for the temporary

inhibition of growth between the fifth and tenth days. The possible effects of this unrecognized pathological state on the outcome of such investigations remain to be determined.

SUMMARY

1. Hemorrhagic degeneration occurred within 10 days in male rats, 20 to 30 days of age, on a low choline diet containing 0.3% of added cystine.

2. A marked decrease in the incidence of the deficiency in similar rats, 33 days of age or older, suggested that there is a corresponding decrease in the choline requirement of rats over 30 days of age.

3. The renal lesions in 40-gm. male rats, 21 to 26 days of age, were most severe on the sixth and seventh days. Only 60 to 80% of these rats survived the 10-day period.

4. The results of choline deficiency appeared more slowly and were less severe in young female rats than in male rats of the same age and weight.

5. The administration of choline after the appearance of hemorrhagic degeneration accelerated the recovery from the acute stage of the deficiency.

6. The increase in weight of the kidneys of male rats, 20 days of age and 24 to 30 gm. in weight, after a 7-day period on diets containing suboptimal levels of added choline was proportional to the severity of the deficiency of choline.

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WHEAT AS A DIETARY SOURCE OF IRON

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ONE FIGURE

It was shown by the classic investigations of Sherman ('32) and it has been emphasized by the work of Rose ('38) that wheat and other cereals are important dietary sources of iron, particularly for people of the so-called low income group. It is well known that refined milling products, so largely used in the diet, contain less iron than the entire grain. The present noteworthy tendency to restore important nutritional essentials to processed foods demands that accurate information about the composition and nutritive value of the unprocessed foods be available. Information about wheat as a dietary source of iron has been conflicting.

The results of a number of published reports on the average iron content of different varieties of wheat are included in the data provided by table 1. Sherman considered that the average iron content of whole wheat is about 5 mg. per 100 gm. Many of the more recent reports indicate that the iron content of wheat is usually between 3 and 4 mg. per each 100 gm. Some variation is to be expected in natural food products but a variation of from 1 to 32 mg. per 100 gm. in wheat as reported by Greaves and Hirst ('29) seems extreme.

The nutritional value of the iron of wheat has been studied by assays with anemic rats and by chemical analyses of so-called available iron. From the rate of increase in the con-

centration of hemoglobin following the feeding of wheat to anemic rats Elvehjem, Hart and Sherman ('33) concluded that the iron of wheat was about half as effective as an inorganic salt of iron. Others have found by similar feeding tests that the iron of wheat is as well utilized as inorganic iron salts (Vahlteich, Rose and MacLeod, '36; Smith and Otis, '37). The chemical method (Hill, '30; Elvehjem, Hart and Sherman, '33) for estimating the nutritional value of the iron of foods also has given conflicting results. The fraction of the total iron which, after treatment with a reducing agent, reacts with dipyridyl has been variously referred to as available, inorganic and ionizable iron. Hahn and Whipple ('38) have criticized the use of the term "available iron" because it is not at once apparent from this expression that 100% availability, for example, means only that the iron of the food is utilized by the body as well as an equal amount of iron in the form of an inorganic salt. "Inorganic iron" and "ionizable iron" are terms which may be criticized on chemical grounds. We believe that a term to be preferred for the iron of foods and tissues which reacts with dipyridyl when treated according to Hill's method, or modifications thereof, is "ionogenic iron." This term, which may be defined according to Shohl's terminology ('39) as the iron which yields ions in the course of body processes, has been adopted in the present report. The values for the ionogenic iron content of wheat included in table 1 emphasize the conflicting nature of the data obtained by chemical methods for the estimation of the nutritive value of the iron of foods. It is not clear from these data whether the iron of wheat is 43 or 92% as efficient as an equal amount of iron in the form of ferric chloride.

The present report provides some additional data on the total and ionogenic iron content on a number of varieties of American grown wheat. Bioassays of the availability of the iron of two samples of wheat are also reported. The data from both studies are discussed briefly with reference to published evidence in the literature.

TABLE 1

Survey of published reports of the average iron content of wheat

REFERENCE	TOTAL FE	IONOGENIC FE	IONOGENIC FE
	mg./100 gm.	*mg./100 gm.*	%
Leach ('09)	3.06		
Sherman ('32)	5.00		
Peterson and Elvehjem ('28)	3.72		
Rose and Vahlteich ('32)	3.30		
Elvehjem, Hart and Sherman ('33)	5.00	2.33	47
Sullivan ('33)	3.10*		
Rose, Vahlteich and MacLeod ('34)	3.16		
Saiki et al. ('34)	3.5		
Vahlteich, Rose and MacLeod ('36)	3.33		
Smith and Otis ('37)	5.18	4.77	92
Aykroyd ('37)	5.37		
Goswami and Basu ('38)	5.5	2.74	50
Ranganathan ('38)	3.97	1.69	43
Free and Bing (present study)	3.94	3.19	81

* Dry basis.

EXPERIMENTAL

Sources of wheat. Under the Official Grain Standards Act of the United States, wheat is divided into five separate classes. These differ in habits of growth, in appearance and in chemical composition and physical properties. We are indebted to Dr. R. M. Bethke of the Ohio Agricultural Experiment Station, Wooster, for three samples of soft red winter wheat (nos. 1, 2 and 3 in table 2) and to the late Dr. Kurt S. Franke of the South Dakota Agricultural Experiment Station, Brookings, for the samples of hard red spring wheat, hard red winter wheat, and durum wheat (nos. 7 to 11 inclusive in table 2). The remaining samples were obtained from local stores (nos. 4 and 5 in table 2) and from the Miami Milling Co., Oxford, Ohio (no. 6 in table 2), and were not identified as to variety, although they belonged to the class of soft red winter wheat. Thus four of the five classes of wheat were included in the present study and, according to Hughes and Henson ('30), the one class not included, soft white wheat, is not grown to any large extent.

Chemical methods. To determine the total iron content a sample of 10 gm. was weighed into a porcelain dish, dried and 0.5 gm. of iron-free calcium acetate added. The sample was

ashed in an electric muffle furnace at from 500° to 550°C., after which the iron was determined by the thioglycolic acid colorimetric method of Hanzal ('33), using the technic described by Bing, Saurwein and Myers ('34).

The determinations of the ionogenic iron content of wheat were carried out on samples which had been finely ground in

TABLE 2

Total and ionogenic iron of wheat

VARIETY AND CLASS	MOISTURE	TOTAL IRON	IONOGENIC IRON	IONOGENIC IRON
	%	mg. per 100 gm.	mg. per 100 gm.	% of total iron
1. Nabob				
Soft red winter wheat	8.3	2.90	2.46	85
2. Fulhio				
Soft red winter wheat	9.6	3.90	3.19	82
3. Trumbull				
Soft red winter wheat	8.2	3.48	2.76	79
4. Unknown (no. 1)				
Soft red winter wheat	10.0	4.87	4.04	83
5. Unknown (no. 2)				
Soft red winter wheat	11.1	3.72	3.23	87
6. Unknown (no. 3)				
Soft red winter wheat	10.3	3.04	2.60	86
7. Turkey				
Hard red winter wheat	7.5	4.20	3.14	75
8. Ceres				
Hard red spring wheat	7.3	3.66	2.95	81
9. Marquis				
Hard red spring wheat	7.5	4.36	3.18	73
10. Acme				
Durum wheat	7.9	4.40	3.85	88
11. Mindum				
Durum wheat	6.6	4.81	3.72	77

a porcelain mortar. The method used was similar to that described by Elvehjem, Hart and Sherman ('33). Because certain modifications have been employed and because of the variation in results obtained by different investigators, the exact technic is described briefly.

A sample of finely ground wheat weighing 3.000 gm. is placed in a round-bottom centrifuge tube, capacity 50 cc. To

the tube are added 5 cc. of M sodium acetate, 2 cc. of 10% acetic acid, and 5 cc. of water, which provides a buffer mixture with a pH about 5.0 for the extraction of the iron. To reduce the iron to the ferrous condition, 0.25 gm. of iron-free sodium hydrosulfite is added. This is followed by a small quantity, about 1 mg., of dipyridyl, which develops a red color with ferrous iron. The contents of the tube are mixed, the tube is stoppered and allowed to stand at room temperature for from 36 to 48 hours with occasional stirring. The longer extraction time is necessary with samples of hard wheat. At the end of this time 5 cc. of 95% ethyl alcohol are added to precipitate soluble proteins and other extracted substances. The tube is then placed in a refrigerator at 5°C. for 12 hours, by which means turbidity of the final solution is avoided. The material is then centrifuged while cold and an aliquot portion of the clear red extract is removed with a pipette.

The amount of iron is determined by colorimetric comparison, using a series of standards in calibrated test tubes supplied with paraffined cork stoppers. The readings are made in a comparator block in daylight, with a white porcelain plate as a background. It is convenient to select an amount of solution so that the readings are made with concentrations of from 0.13 to 0.16 mg. of iron per 100 cc.

The standards are prepared from a solution of 0.0001 M iron dipyridyl in 0.2 M sodium acetate-acetic acid buffer, with an excess of dipyridyl and sodium hydrosulfite. A series of tubes providing a range of from 0.070 to 0.300 mg. of iron per 100 cc. was made. In all determinations a blank was run to allow for the yellow colored material extracted from the wheat. The blank is prepared by weighing out 3.000 gm. of the sample and treating this exactly the same as the sample being analyzed, except that no dipyridyl is added. This control tube is placed behind the standard tube and a tube of water is placed behind the "unknown" tube when colorimetric comparisons are made.

Recovery experiments with added iron gave theoretical values within ±5%. Longer extraction did not increase the

amount of ionogenic iron obtained from any of the wheat samples. In order to ascertain whether prolonged extraction might have a destructive effect on organic iron compounds, hematin was added to samples of wheat. Although the amount of iron in the form of hematin which was added was equal to twice the iron content of the wheat, there was no change in the apparent ionogenic iron as determined by the present method.

The results of the determinations of total and ionogenic iron in eleven specimens of wheat as determined by the methods described are provided in table 2. This table shows that the total iron varied from 2.90 to 4.87 mg. per 100 gm. of wheat and the ionogenic iron from 2.46 to 4.04 mg. per 100 gm. of wheat. The averages for all samples, regardless of variety, were 3.94 mg. of total iron and 3.19 mg. of ionogenic iron per 100 gm. of wheat. The variations in iron content appear to be unrelated to the variety of the wheat. The ionogenic iron expressed as a percentage of the total iron varied from 73 to 88%, and the average value was 81%. The ionogenic iron appears to be a relatively constant proportion of the total iron. As a result, there were some samples having a high total iron content, in which the ionogenic iron was considerably higher than the total iron of samples of wheat having a relatively low iron content.

The nature of the organic iron of wheat has not been determined. Wheat gives a positive test with benzidine and hydrogen peroxide which may indicate the presence of a hematin compound. The possibility that even most of the residual iron is present in some simple inorganic combination which is not extracted by the technic employed cannot be denied.

The results of the present study are not in agreement with those of Elvehjem, Hart and Sherman ('33) or of two investigators in India (Goswami and Basu, '38; Ranganathan, '38) who found that only about one-half of the iron in wheat is available by chemical tests. Our results are somewhat lower than those of Smith and Otis ('37), who found by the dipyridyl method that 92% of the total iron of wheat is ionogenic. When

Myers, Remp and Bing ('35) first tested the "availability" of iron in bread by the dipyridyl method they found only about 50% of the total iron to be ionogenic. Later studies showed that with a longer period of extraction, from 94 to 97% of the total iron in white, rye and whole wheat bread is ionogenic (Remp, '35). McCance ('39) likewise has called attention to discrepancies in the values recorded for the ionogenic iron content of foods by different workers.

Biological assay. Although the chemical method for the estimation of the nutritional value of the iron of foods may become acceptable, there is no doubt that the figures obtained at the present time not only are apt to vary with different technics but also the results are difficult to interpret. The bioassay of the iron of foods is tedious and time-consuming, but it remains the best method for the purpose of determining whether the iron in a food product can be utilized by the body. Rose and Vahlteich ('32) have shown by feeding experiments with anemic rats that the iron of wheat is as well utilized as the iron of ferric chloride. Smith and Otis ('37) likewise have reported that the iron of wheat is well utilized. Elvehjem, Hart and Sherman ('33), however, reported that, as a source of iron, wheat is about half as valuable as iron in the form of a simple inorganic salt. Vahlteich, Rose and MacLeod ('36) have presented evidence indicating that the iron of wheat which has been treated with diastase is even better utilized than an equal amount of iron in the form of an iron salt. Remp ('35) also has found that when wheat is fed in the form of bread, the iron is somewhat better utilized than an equal amount of iron in the form of ferric chloride.

In the present experiments assays were made of the iron value of two varieties of soft red winter wheat. Briefly, the technic was as follows: Albino rats were made anemic by restriction to a milk diet from the time of weaning. Raw Guernsey milk obtained by milking directly in glass bottles was used. When the hemoglobin level of the blood was below 4.0 gm. per 100 cc., the animals were placed on the experimental diets. One group of five animals served as controls and each

rat in this group was given daily 0.25 mg. of iron in the form
of ferric chloride. A group of six animals received sufficient
Trumbull wheat (a soft red winter wheat) to supply daily
0.25 mg. of iron to each rat. Another group of six animals
received Nabob wheat (a soft red winter wheat) in quantities
sufficient to provide 0.25 mg. of iron per day. No difficulty was
experienced in securing complete consumption of the amounts
of wheat necessary to provide the selected dosage of iron. The
rats in all three groups were given daily supplements of 0.05
mg. of copper as copper sulphate and 0.05 mg. of manganese
as manganous chloride. All animals received as much milk as
they would drink, following the consumption of the daily wheat
ration. The food intake of each animal was measured and in
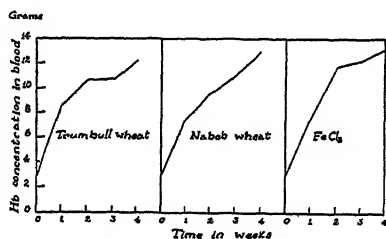
Fig. 1   The average hemoglobin concentration, as grams per 100 cc. of blood,
is shown for anemic rats which received daily 0.25 mg. of Fe as wheat or FeCl₃.

that way the intake of iron was calculated. The animals grew
well for the duration of the experiment and there was no
particular difference in the response of male and female ani-
mals. Each group contained, whenever possible, equal num-
bers of males and females. An examination of other data
obtained in our laboratory shows that if there is any difference
in the response of males and females it is very small, even for
animals receiving doses of iron smaller than 0.25 mg. daily.
Perhaps this is because no difference in the food consumption
of males and females was observed during the short time of
the feeding test.

The hemoglobin concentration of the blood was determined
weekly during the 4-week period. The average values for the
three groups of animals are plotted in figure 1. It may be

mentioned that the response of the animals receiving the ferric chloride was similar to that of many other animals in our colony which had received similar treatment. The average increase of hemoglobin in the rats receiving ferric chloride was 10.1 gm. per 100 cc. of blood. The rats receiving Nabob wheat showed an increase of 10.0 gm. and the rats receiving Trumbull wheat 9.1 gm. of hemoglobin per 100 cc. of blood. The increase in hemoglobin in the three groups of animals is considered to be essentially the same.

Although the response in hemoglobin concentration is usually taken as a measure of the availability of iron, we have found it desirable to supplement the data by analysis of the animals for iron content. A discussion of the methods of assay for iron need not be made in the present report. Briefly the method is as follows: At the end of the experiment, in this case at the end of 4 weeks, the animals are killed by prolonged anesthesia with ether. The carcasses are then cleaned, washed with distilled water, and placed in the refrigerator for several hours. The stomach and intestines are then dissected and removed and the rest of the carcass is ashed in an electric muffle furnace. The method of analysis has already been described (Bing, Saurwein and Myers, '34).

The retention of iron is determined by subtracting from the iron content of the body the amount estimated to have been present at the beginning of the experiment. The initial iron content is calculated from analyses of the bodies of control animals having a similar nutritional history, the same weight and hemoglobin concentration in the blood. In table 3 are shown the results of this method of determining the retention of the iron. The calculated initial content of iron was on the average in each group 1 mg., or a little more, and after receiving the indicated treatment for 4 weeks the iron content had increased by from 3.3 to 3.4 mg. The percentage utilization is determined by multiplying the value for the retention of iron by 100 and dividing by the estimated iron intake during the experimental period. As shown in the table, there was a slight variation in the total iron intake, depending on spillage

of food, consumption of milk, and such factors, all of which were taken into account. The percentage utilization of the iron of ferric chloride under the conditions of the experiments was 47% of the intake. In the wheat 48% of the iron of one sample was retained and 51% of the iron of the other sample. These data show clearly that the iron of wheat is just as well utilized as an equal amount of iron in the form of ferric chloride.

TABLE 3

Results of biological assay of the availability of the iron of wheat

SOURCE OF IRON	TOTAL IRON INTAKE	CALCULATED INITIAL BODY IRON	FINAL BODY IRON	INCREASE IN IRON	RETEN-TION
	mg.	*mg.*	*mg.*	*mg.*	*%*
Ferric chloride (average of 5 rats)	7.29	1.00	4.39	3.39	47
Trumbull wheat (average of 6 rats)	7.01	1.10	4.43	3.33	48
Nabob wheat (average of 6 rats)	6.80	1.03	4.48	3.45	51

SUMMARY AND CONCLUSIONS

The iron content of eleven samples of American grown wheat varied from 2.90 to 4.87 mg. per 100 gm., and the average was 3.94 mg. These values are in accord with most of the recent reports of the iron content of wheat, which show that about 3 to 4 mg. of iron are present per 100 gm. It was shown by the dipyridyl method that an average of 81% of the total iron of wheat is ionogenic iron (also called available and inorganic iron). The proportion of the total iron in the form of ionogenic iron in the present series of determinations ranged from 73 to 88%. Within these limits the ionogenic iron is a constant proportion of the total iron. By means of biological assays with anemic rats it was shown that the iron of wheat is fully as available to the organism as an equal amount of iron in the form of ferric chloride. Under the conditions described, about one-half of the iron of ferric chloride or of finely ground whole wheat is retained by the anemic rat. In view of the results of

the feeding tests it is difficult to interpret the values obtained with the chemical method for the estimation of ionogenic iron.

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EFFECTS OF SIMPLE DIETARY ALTERATIONS UPON RETENTION OF POSITIVE AND NEGATIVE MINERALS BY CHILDREN [1]

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The rapidly developing science of nutrition has demanded specific knowledge concerning the physiologic effects of individual foods as they undergo the various phases of digestion, absorption, and utilization. Frequently, in dietetic practice, one food is substituted for another on the basis of a single food principle without regard for equally important component parts of the diet. It is essential that consideration be given to the physiologic events consequent to the changed characteristics of the mixed diet as a result of altering the quantity of only one food, which in itself is subject to wide variations in composition. Isocaloric diets and the total nitrogen of diets have been studied, but little is known of the minerals in diets beyond some effects of different levels and proportions of calcium and phosphorus upon metabolism.

No two natural foods are exactly alike; their caloric values may be identical while their carbohydrates vary in availability and the indigestible portions possess different physical characteristics. Likewise, with similar calorigenic qualities their mineral contents may be unlike and the various minerals present in dissimilar proportions; the total calorie and nitrogen

461

contents may be similar but the various vitamins may not be present in effective proportions; they may have the same total nitrogen content while the proportions of the different amino acids in the proteins are so different that one food produces nutritive success and another failure. Positive knowledge of the physiologic effects produced by dietary alterations is needed. The nutritive value of these effects must be determined if dietetic requirements are to be adequately provided for during growth of the child and maintenance of the adult, in both health and disease.

The present investigation, only one phase of an extensive study of growth, deals with the effects of simple dietary alterations upon retention of positive and negative minerals by normal, active children. The intakes of positive and negative minerals were maintained at approximately constant levels for each subject but the distribution of component inorganic elements within the positive (calcium, magnesium, sodium and potassium) and negative (phosphorus, sulfur and chlorine) mineral groups were varied during the experimental periods by altering the proportions of banana (an alkaline-ash food with a high concentration of potassium), apple, and cereal in adequate mixed diets. During both the pre-experimental and experimental periods the alkaline-ash values were sufficient for growth needs (Shohl, '23) although the values were approximately doubled during the latter.[2]

<center>METHODS</center>

Nine children, 5 to 8 years of age (four girls and five boys), who through intensive physiologic and medical observation were known to be in favorable nutritive condition, were the subjects of metabolic study during pre-experimental and ex-

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[2] The recent trend toward greater accuracy in referring to electropositive and electronegative elements or radicals has prompted the use of terms based upon the nomenclature used by Shohl ('39) in his monograph on ''Mineral Metabolism.'' Former use of the words ''base'' and ''acid'' has been replaced by ''positive minerals, or cations'' and ''negative minerals or anions.'' ''Ion balance'' or ''excess ions'' has replaced acid-base balance for urine, feces, and retentions and alkaline-ash value is used for the ''excess of positive minerals'' in intakes.

perimental periods ranging from 20 to 55 consecutive days for each child, a total of 640 days. The Methodist Children's Village afforded ideal facilities for metabolic observations. The children lived together in a home unit with a housemother; went to school and to Sunday school in the Village; enjoyed their customary freedom of play out of doors with pets and playthings; and were given every opportunity for unhampered social and physical development. They slept in individual beds in pleasant airy rooms, and were at all times, even going to and from school, under the continuous observation of a trained person, so it is known that no metabolic samples were lost or extra foods eaten. Three young women, trained in nutrition and dietetics, quantitatively prepared and served the food and collected all the metabolic specimens which were analyzed [3] by the staff of the Research Laboratory of the Children's Fund of Michigan. Two housemothers gave special attention to the emotional care and management of the children.

The children's diets were composed of twenty-one common foods [4] and conformed to accepted dietetic practice, providing averages of approximately 0.8 gm. of calcium and 60 gm. of protein per child per day, in addition to ample amounts of vitamins. They were comparable qualitatively but quantity was adjusted to meet the requirements of the individual's age, size and activity. Each subject served as his own control, for growth and response to a given dietary regimen differ among children, owing to dissimilar inheritance and treatment during growth and development. Each child's gastrointestinal pattern (Macy, Reynolds, Bates and Souders, '40) and response to different types of test meals were known (Reynolds, Macy and

[3] The food, urine, and feces were each analyzed for nitrogen, calcium, magnesium, sodium, potassium, phosphorus, sulfur, and chlorine. The chemical methods, omitted by space requirements, may be obtained from the authors. Methods of analysis will be presented in detail in a forthcoming monograph upon the chemical growth of children.

[4] The mixed diet contained the following foods: apple, banana, lean beef, white and whole wheat bread, shredded wheat, cabbage, carrots, American cheese, eggs, lettuce, milk, orange juice, peanut butter, potato, spinach, tomato juice, graham crackers, butter, sugar and salt.

Bates, '40). The health status,[5] care, and management of the children, procedures, and chemical methods are detailed elsewhere (Hunscher, Hummel and Macy, '39; Hummel, Hunscher and Macy, '39).

Banana was selected as the basis of the dietary manipulations because the fruit is generally liked (Alvarez and Hinshaw, '35) and has been proved beneficial to well infants (Johnston, '27; von Meysenbug, '27), those with debilitated digestive disorders (Haas, '32; Brubaker, '37) and scurvy (Johnston, '27) and the development and well-being of children (Brown and Courtney, '29; Roberts, Blair, Austin and Steininger, '39). In addition, the ash of banana is high in potassium, low in calcium, and, when 100 gm. were added or substituted for cereal in the daily mixed diet, its high alkaline-ash value permitted an increase of approximately 50% in the alkaline-ash values of the intakes per kilogram of body weight without altering the total minerals content, even though the pre-experimental mixed diets contained different amounts of apple and banana. Seven children were already receiving both apple and banana in their mixed diets, eliminating the possibility of effects from intolerance or allergy in the results.

The alterations made in the diets of eight subjects during the experimental period consisted of the addition or substitution for cereal of 100 gm. of banana. One subject had other additions (10 gm. white bread; 20 gm. cereal) to the diet during the experimental period. Five of the eight children were receiving identical amounts of raw apple and raw banana combined throughout the pre-experimental and experimental periods, 200 gm. daily during the pre-experimental period and 300 gm. during the experimental period. However, three children received 100 and two subjects 200 gm. of apple daily during the pre-experimental period; the former receiving 100 gm. of banana per day and the latter receiving no banana. This variation in the mixed diets of the five children produced a difference between the proportionate compositions of the

diets which was accentuated during the experimental period even though the changes in dietary were the same.

The effect of each variation upon the mineral composition of the food intakes is shown in table 1. It is important to note that the addition or substitution of 100 gm. of banana produced an increase of approximately 7.7 meq. in the alkaline-ash value of the intakes regardless of other differences in the diets. When added to the mixed diet, 100 gm. of banana increased the intake of total minerals 18.1 meq.; when substituted for cereal the intake was only increased by 2.0 meq. In relation to the quantities of total minerals in the mixed diets these changes are small as is the difference in total minerals between 100 gm. of banana and 100 gm. of apple (12.9 meq.). Per kilogram of body weight these changes become even less significant, although the conservative changes did cause significant and wide variations in the proportions of the different individual elements in the mixed diets (Hunscher, Hummel and Macy, '40). These variations in individual elements, significant in themselves, did not produce significant differences in the intakes of total positive or total negative minerals per kilogram of body weight (table 2). During the pre-experimental period the subjects' intakes (table 2) provided an average daily excess of 0.67 meq. of positive minerals per kilogram of body weight. This was increased 0.43 meq. (0.67 to 1.10 meq.) per kilogram of body weight during the experimental period, making the average mineral intakes 1.10 meq. per kilogram of body weight. These amounts are comparable with the excess of 10 cc. of 0.1 N base (1.0 meq.) which Shohl ('23) considers necessary for growth.

Banana and apple contain about 19 and 12% available carbohydrate, respectively (Widdowson and McCance, '35), and have other beneficial dietary properties that are not fully understood (Bergeim, Hanszen and Arnold, '36; Baumann and Forschner-Böke, '34; von Loesecke, '30). Apple and banana contain similar amounts of "unavailable" carbohy-

TABLE 1

Average daily chemical variations in diets consequent to variations in their banana, apple and cereal contents

	PRE-EXPERIMENTAL PERIOD MIXED DIET [1] (Group I)	ADDITION OF 100 GM. BANANA (All groups)	DELETION OF CEREAL [2] (Groups II, III)	CHANGES [3] DUE TO SUBSTITUTION (Groups II, III)	CHANGES IN ADDITION TO BANANA (Group IV)	VARIATION DUE TO 100 GM. APPLE (Groups II, III)
Total calories	1900	99	93	+6	255	63
Nitrogen (gm.)	10.3	0.1	0.4	—0.3	0.9	0.1
Protein (gm.)	64.5	0.7	2.3	—1.6	5.4	0.4
Fat (gm.)	69.5	0.1	0.6	—0.5	15.4	0.2
Total minerals (meq.)	419.2	18.1	16.1	+2.0	41.8	5.2
Positive minerals (meq.)	217.3	12.9	8.1	+4.8	21.5	4.1
Calcium (meq.)	38.3	0.2	0.7	—0.5	2.2	0.2
Magnesium (meq.)	21.9	2.7	0.8	+1.9	1.5	0.6
Sodium (meq.)	94.6	0.2	5.1	—4.9	13.0	0.1
Potassium (meq.)	62.5	9.8	1.5	+8.3	4.8	3.2
Negative minerals (meq.)	201.9	5.2	8.0	—2.8	20.3	1.1
Phosphorus (meq.)	62.5	1.6	3.1	—1.5	4.6	0.8
Chlorine (meq.)	96.2	3.2	3.0	+0.2	10.7	0.1
Sulfur (meq.)	43.2	0.4	1.9	—1.5	5.0	0.2
Alkaline-ash value (meq.)	15.4	7.7	0.1	+7.6	1.2	3.0

[1] The caloric value of the diets varied with intakes of sugar and diet levels were adjusted to age, size, and activity of the individuals. The diet value given is for D.P. All data are from analyses.

[2] Ten grams of white bread and 20 gm. of shredded wheat or corn flakes.

[3] Change in content when 100 gm. of banana were substituted for cereal.

drate but their proportionate compositions of lignin, cellulose and hemicellulose are dissimilar. These variations and their influence upon the disappearance of complex carbohydrates from the tracts of these children have been discussed (Hummel, Shepherd and Macy, '39; Shepherd, Hummel and Macy, '40). It is known that banana and apple influence the bactericidal power of the stomach (Hanszen, '34) and that pectin (Manville, Bradway and McMinis, '36; Ziegelmayer, '36) and the flora of the intestine (von Meysenbug and Fine, '36) influence nutritional processes. Myers and Rose ('17) found the reducing sugar in the very green, medium ripe, and very ripe banana to be 0.55, 2.88 and 7.08%; the non-reducing sugar (sucrose), 1.20, 12.52 and 11.10%; and the total sugar, 1.75, 15.40 and 18.18%, respectively. The bananas used in this investigation were fed when in a state of optimum [6] ripeness, and the apples were of the same variety and all purchased from the same source.

Ages, recumbent lengths, weights, the average daily caloric intakes, and nitrogen and mineral ion balances per kilogram of body weight for the nine children are presented in table 2. The average intakes per kilogram of body weight for the nine children show, for the experimental period, a slight increase in caloric value (2 cal.) and a reduction in nitrogen (10 mg.). In order to attain proportionate growth and development with a satisfactory distribution of the essential chemical elements in the skeletal, muscular, glandular and neural tissues, it is of synergistic nutritive advantage to have a continual storage of both positive and negative minerals, with a preponderance of the former (Shohl, '39; Hunscher, Hummel and Macy, '39). The subjects stored, on the average, 0.70 meq. of cations and 0.57 meq. of anions, a total of 1.27 meq. of minerals daily per kilogram of body weight during the pre-experimental period, the quantities increasing during the experimental period 0.20, 0.29 and 0.49 meq., respectively, which shows that, subject to individual variation, tissue synthesis was occurring through-

[6] Carefully selected bananas were furnished by an experienced wholesaler, Mr. B. E. Reiff, Fruit Dispatch Company, Detroit.

TABLE 2

Average daily balances of nitrogen, and positive and

| | | OBSER-VATION PERIOD | INITIAL AGE | RECUM-BENT LENGTH | WEIGHT | DIETARY CHANGES [1] | | TOTAL ENERGY INTAKE |
						Raw apple	Raw banana	
		days	*mo./days*	*cm.*	*kg.*	*gm./day*	*gm./day*	*Cal./kg.*
Group I:								
H.H.	Pre-experimental	35	84/24	119.8	20.76	100	100	90
	Experimental	40	85/28	120.2	21.33	100	200	93
F.C.	Pre-experimental	35	79/ 5	119.4	21.60	100	100	87
	Experimental	40	80/ 9	120.1	22.40	100	200	88
D.P.	Pre-experimental	35	98/20	136.0	27.68	100	100	69
	Experimental	30	99/24	137.1	27.60	100	200	72
Group II:								
R.S.	Pre-experimental	30	65/17	114.0	18.26	100	100	90
	Experimental	20	66/21	114.7	18.40	100	200	89
B.M.[3]	Pre-experimental	30	63/ 7	104.1	16.23	100	100	100
	Experimental	30	64/ 6	105.3	16.88	100	200	96
J.H.	Pre-experimental	30	55/22	106.9	16.84	100	100	97
	Experimental	30	56/21	107.4	16.87	100	200	97
Group III:								
J.M.[3]	Pre-experimental	40	75/ 3	116.4	20.64	200	——	85
	Experimental	45	76/17	117.4	21.58	200	100	82
B.F.[3]	Pre-experimental	40	69/12	110.5	18.43	200	——	95
	Experimental	50	70/21	111.4	19.28	200	100	91
Group IV:								
P.W.[3]	Pre-experimental	55	75/29	116.8	20.21	100	100	80
	Experimental	25	78/ 3	118.2	21.15	100	200	93
Pre-experimental period of		330 days:					Mean ±SE$_m$[6]	87
Experimental period of		310 days:					Mean ±SE$_m$[6]	89

[1] For groups II and III the 100 gm. banana added to the mixed diet during the experimental period was a substitution for 10 gm. white bread and 20 gm. cereal (shredded wheat or corn flakes) removed from the dietary. For group IV in addition to 100 gm. banana, 30 gm. potato, 10 gm. butter, and 50 gm. white bread were added to the daily diet during the experimental period.

[2] Sum of values for calcium, magnesium, sodium, potassium, phosphorus, chlorine and sulfur.

[3] Valences of 1.8 for phosphorus and 2 for sulfur have been used in calculations.

TABLE 2

negative minerals in response to altered diets

NITROGEN		MINERAL ASH-VALUES OF INTAKES				ION BALANCES IN EXCRETION[4]		RETENTION OF MINERAL IONS			
Intake	Reten-tion	Total[2]	Posi-tive	Nega-tive[3]	Alkaline ash values	Urine	Feces	Total[2]	Cations	Anions[3]	Excess ions[4]
mg./kg.	mg./kg.	meq./kg.	meq./kg.	meq./kg.	meq./kg.	meq./kg.	meq./kg.	meq./ky.	meq./kg.	meq./kg.	meq./kg.
497	4	20.19	10.46	9.73	0.73	0.62-	1.21+	1.14	0.64	0.50	0.14+
489	20	20.50	10.79	9.71	1.08	0.04+	1.24+	1.72	0.76	0.96	0.20-
478	4	19.41	10.06	9.35	0.71	0.67-	1.25+	1.18	0.66	0.52	0.13-
466	18	19.51	10.27	9.24	1.03	0.02+	1.31+	1.86	0.78	1.08	0.30-
373	2	15.14	7.85	7.29	0.55	0.48-	0.84+	0.81	0.50	0.31	0.19+
378	2	15.84	8.34	7.50	0.84	0.39-	1.06+	1.61	0.89	0.72	0.16+
517	43	20.65	10.71	9.94	0.77	0.89-	1.36+	1.52	0.91	0.61	0.30+
503	19	20.47	10.97	9.50	1.47	0.61-	1.68+	1.00	0.70	0.30	0.40+
582	27	23.23	12.05	11.18	0.87	0.72-	1.49-	1.64	0.87	0.77	0.10+
549	26	22.32	11.96	10.36	1.60	0.36-	1.75+	1.87	1.04	0.83	0.21+
561	13	22.39	11.61	10.78	0.84	0.75-	1.33+	1.16	0.71	0.45	0.26+
549	10	22.34	11.97	10.37	1.60	0.38-	1.28-	1.82	1.26	0.56	0.70+
498	24	19.59	10.03	9.56	0.47	0.50-	1.15+	1.44	0.63	0.81	0.18-
464	29	18.82	9.81	9.01	0.80	0.26-	1.08+	1.72	0.85	0.87	0.02-
558	20	21.94	11.23	10.71	0.52	0.83-	1.42+	1.75	0.84	0.91	0.07-
520	36	21.07	10.98	10.09	0.89	0.42-	1.34+	1.81	0.89	0.92	0.03-
468	—6	18.66	9.68	8.98	0.70	0.67-	1.11+	0.94	0.60	0.34	0.26+
495	38	20.77	10.93	9.84	1.10	0.12+	1.23+	2.15	0.95	1.20	0.25-
500	13	19.98	10.33	9.65	0.67	0.68-	1.23+	1.27	0.70	0.57	0.12+
±7	±3	±0.27	±0.14	±0.14	±0.02	±0.04	±0.04	±0.08	±0.05	±0.05	±0.05
490	23	20.15	10.63	9.52	1.10	0.24-	1.31+	1.76	0.90	0.86	0.04+
±6	±2	±0.23	±0.12	±0.10	±0.04	±0.04	±0.03	±0.07	±0.04	±0.04	±0.05

[4] Excess of anions is indicated by a superior ''minus'' sign following the figures; excess of cations by a superior ''plus'' sign.

[5] Girls.

[6] SE_m represents the standard error of the mean and is the standard deviation divided by the square root of the number of observations.

out the observation, though at a greater rate during the latter period.

It is evident that the amounts and proportions in which the different elements are deposited in the body either determine or are determined by the type of construction occurring in the organism at a given time; thus, quantity intake or proportion may control the retention of minerals but many additional influences are also operating. Muscular, glandular, and neural tissues may be forming at a much greater rate at one time and skeletal tissue, containing the major amounts of the body's basic elements, at another. For this reason inquiry must be made into the reaction of the individuals to the addition of banana, to the substitution of banana for cereal, and to the substitution of banana for cereal under two different conditions owing to varied proportions of raw apple and raw banana in the total mixed diets.

The three children (group I) who received an additional 100 gm. of banana in their mixed diet (200 gm. total) during the experimental period showed average increases in daily retention of nitrogen (3 to 14 mg.), cations (0.6 to 0.8 meq.), anions (0.44 to 0.94 meq.), and total mineral ions (1.04 to 1.74 meq.) per kilogram of body weight. It is significant that the supplementary fruit enhanced the nutritive advantage of the diet in spite of the fact that the nitrogen intakes were reduced for two of the three children. With their nitrogen intakes lowered 8 and 12 mg. per kilogram of body weight, their storage of nitrogen increased from 4 to 20 and 18 mg. per kilogram, respectively. Since the accumulation of nitrogen is a good index of the growth of muscle and soft tissues it is evident that all of the children were constructing new tissue but the rate was greater during the experimental period in spite of a reduced nitrogen intake in two of the subjects (H.H. and F.C.). Although there was no weight increase by the third child (D.P.) the small amount of nitrogen stored was no doubt used in the building of the organic matrix in skeletal growth.

Daily energy intakes were only increased 1 to 3 calories per kilogram of body weight during the experimental period, a

quantity which would not greatly influence nitrogen metabolism. It is of significance to note that all three subjects (group I) gained in height and weight with the exception of D.P., who lost weight during the experimental period although he did store some nitrogen. However, his increase in height exceeded the rest of the group. The fact that there was augmented rate of growth during the experimental period is further verified by the storage of total mineral ions by all three subjects, the quantity being doubled for D.P., whose gain in height was greatest. Furthermore, on a unit weight basis these children in group I all retained an excess of cations during the pre-experimental period (13–19 meq.) but when additional banana was given H.H. and F.C. increased particularly their rates of soft tissue synthesis, indicated by greater retentions of nitrogen and retention of excess anions (0.20 and 0.30 meq. daily). D.P. continued to store a large excess of cations (0.16 meq.) daily per kilogram of body weight, 80% of which were used for skeletal growth, additional evidence that structure composition predominated over soft tissue growth in this child during the study.

The controlling factor in the different responses of H.H. and F.C., which indicated a stimulation of soft tissue growth, and that of D.P., which indicated accelerated skeletal growth, is evident in the ion balances for the urine and feces. The urine of H.H. and F.C. contained excesses of 0.62 and 0.67 meq. of anions, respectively, during the pre-experimental period. These excesses were changed to 0.04 and 0.02 meq. of cations during the experimental period, whereas the excess anions in the urine of D.P. were only reduced from 0.48 to 0.39 meq. In the feces H.H. and F.C. only increased their excretion of excess cations from 1.21 and 1.25 to 1.24 and 1.31 meq., respectively, but D.P.'s excretion increased from 0.84 to 1.06 meq. per kilogram per day.

Substitution of 100 gm. of raw banana for 30 gm. of cereal (10 gm. white bread; 20 gm. shredded wheat or corn flakes) in the mixed diets of the five children in groups II and III produced decreases in the daily nitrogen intakes per kilogram of

body weight which were accompanied by decreased retentions of nitrogen for the three children in group II, and increased nitrogen retentions for the children in group III. On a unit weight basis, with the total mineral ash values of the intakes little changed but with their alkaline-ash values approximately doubled, all five children continued to store mineral ions and four increased their rate of retention. One child (R.S.) who showed by far the largest reduction in rate of nitrogen retention of the three children in group II showed a slower rate of mineral accumulation during the experimental period.

Together, the five children responded similarly to the dietary substitution in their retention of positive and negative minerals. With the exception of the one subject whose rate of storage of total mineral ions was decelerated, the children increased their retentions of both cations and anions. In all five cases the retention of cations in relation to anions was increased, the rate of retention of an excess of cations was increased for all three children in group II and the rate of retention of excess anions was reduced for both children in group III. The increased storage of excess cations by the children in group II, together with reduced nitrogen retentions, indicates a greater effect of the altered diet toward skeleton development. While the children in group II were storing nitrogen and an excess of cations during the pre-experimental period, the subjects in group III were storing an excess of anions, indicating that they were forming a preponderance of soft tissue. The dietary change produced a reduction in these retentions but the excess retentions continued to be of anions and the nitrogen retentions increased. Apparently these differences from the response of group II were due, at least in part, to the variations in the apple and banana contents of the two groups, making the nutritive advantage of the substitution greater for the children who were receiving no banana and 200 gm. of apple during the pre-experimental period. This interpretation is strengthened by the ion balances in the excretion. All of the children in both groups reduced their excretion of an excess of anions in the urine and those

in group III reduced their excretion of excess cations in the feces; however, two of the children in group II (including R.S., whose retention of total minerals was reduced) showed considerable increases in fecal excretion of excess cations and the other child little change. These metabolic responses and changes in growth are paralleled by the changes in body weight and recumbent length.

One child (group IV) received an addition of 100 gm. of banana, 10 gm. of butter, 30 gm. of potato and 50 gm. white bread during the experimental period. The increases in intakes of cations and anions on a unit basis were approximately equal but the retention of anions increased more than twice that of cations during the experimental period, producing a shift in total retention from an excess of cations to an excess of anions. During the pre-experimental period this child showed an average loss of 6 mg. of nitrogen per kilogram of body weight per day; during the experimental period there was an average daily retention of 38 mg. per kilogram. While the data clearly show the effects of the increased intake they also indicate that the characteristics of the changed intake contributed the necessary elements for both types of tissue construction. The chemical composition of growth and the kind of nutrition were very different in the two periods and demonstrate what extensive adjustments a healthy child can make in response to a change in diet.

From the responses of the children it is evident that there are factors other than the quantity intake of positive and negative minerals and the proportion between the ions consumed in the food which influence the type of tissue formation and storage of positive or negative minerals. Moreover, they demonstrate that selective retentions take place in spite of caloric value, mere quantity intakes of nitrogen, positive and negative minerals, or the acid- or alkaline-ash values of the diet and therefore may determine the type of growth present at any one time (i.e., soft or hard tissue formation). Explanations for the type of response that a child gives to a specific dietary change must be sought in the effects of altering the interrela-

tionship of individual mineral constituents or other components of the diet at the same time intakes of nitrogen, cations, anions, and excess ions are varied. The proportion of the different positive (calcium, magnesium, potassium, sodium) and negative (phosphorus, sulfur, and chlorine) mineral elements in the diet may have more importance in metabolism during growth than total quantities of cations, anions and excess ions; or perhaps the growth impulse inherent in the individual may govern the type of tissue formation at any one time rather than the acid-ash and alkaline-ash values of the diet. The inclusion of more banana in the diet did enhance nitrogen, cation and anion retentions, results which cannot be explained on the basis of the quantity intake of these constituents, or by the proportions of excess alkaline-ash consumed, results which corroborate the observations of Roberts and associates ('39).

SUMMARY

1. Nine children, 5 to 8 years of age (four girls and five boys), who, through intensive physiologic and medical observations, were known to be in favorable nutritive condition, were the subjects of metabolic study during pre-experimental and experimental periods ranging from 20 to 55 consecutive days for each child, a total of 640 days.

2. Chemical analyses demonstrate that a conservative substitution of such foods as apple, banana, and cereal in an otherwise constant daily mixed diet on the basis of either the total mineral ash value, the total positive minerals, the total negative minerals, or even doubling the alkaline-ash values, cannot be effected without changing the proportions of other equally important dietary components such as calories, nitrogen, fat, and the proportions of the inorganic elements within the positive or negative mineral groups, any one of which might change the trend of metabolism and subsequent retentions.

3. One hundred grams of banana (one medium sized) was more effective than the same amount of apple or 30 gm. cereal

in stimulating the rate of growth as judged by increases in the individuals' height and weight, and retentions on a unit weight basis of grams nitrogen and milliequivalents of total mineral ions. This growth performance was accomplished in spite of the maintenance on a unit weight basis of approximately a constant daily intake of total positive and negative minerals during both the high and low banana periods, and an alkaline-ash value for the intakes almost doubled during the experimental period. In some subjects the type of growth stimulated was toward an equal development of bony and soft tissues; in others the trend was stronger toward soft tissue (increased storage of nitrogen with either an increased retention of anions or a reduction in retention of cations); and in others greater skeletal formation was initiated (increased storage of cations).

4. Since the dietary changes did not significantly alter the total mineral intakes, these results verify Shohl's ('39) thesis that ". . . . individual elements, or rather, certain groups of elements, perform separate functions in the body economy, and are therefore more important than the total."

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FURTHER STUDIES ON THE EFFECTIVENESS OF ARSENIC IN PREVENTING SELENIUM POISONING [1]

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Recent work at this laboratory (Moxon, '38; Moxon and DuBois, '39) has shown that arsenic will counteract selenium poisoning. It is the purpose of this paper to report further research along this line. This work was carried out on rats as an extension of that already reported.

It has been concerned mainly with the effectiveness of different forms of arsenic in preventing the toxic action of selenium in the form of seleniferous wheat and the effectiveness of arsenic as sodium arsenite in preventing the toxicity of different forms of selenium.

Different forms of arsenic

The effectiveness of different arsenic compounds was compared in controlled experiments with albino rats. The diet used and the management of the animals has been described in a previous publication (Moxon and DuBois, '39). Arsenic compounds were fed according to table 1.

Sodium arsenite (Na_2HAsO_3) and sodium arsenate (Na_2HAsO_4), when used as sources of arsenic, were equally effective in preventing the toxic action of the selenium in the diet (groups 3 and 4 of table 1) as indicated by growth, mortality rate and pathological condition of the livers.

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[1] Approved for publication by the Director of the South Dakota Agricultural Experiment Station as contribution no. 126 of the Journal Series.

Arsenic at this level (5 p.p.m. in the drinking water) did not give full protection against the 14 p.p.m. of selenium in the diet. It has been shown, however, that arsenic at this level will protect rats against 11 p.p.m. of selenium in the diet (Moxon and DuBois, '39), and 10 p.p.m. of arsenic as sodium arsenite protected the rats in group 9 against 18 p.p.m. of selenium in the diet as is shown in figure 1.

In group 5 (table 1) the 10 p.p.m. of arsenic as sodium arsenite, mixed in the diet, prevented the development of symptoms of selenium poisoning slightly better than 5 p.p.m.

TABLE 1

Effectiveness of different forms of arsenic against selenium found in wheat

GROUP OF RATS	SELENIUM USED (IN DIET)	ARSENIC USED
1	14 p.p.m. (seleniferous wheat)	None
2	14 p.p.m. (seleniferous wheat)	None
3	14 p.p.m. (seleniferous wheat)	5 p.p.m. as Na_2HAsO_3 in drinking water.
4	14 p.p.m. (seleniferous wheat)	5 p.p.m. as Na_2HAsO_4 in drinking water.
5	14 p.p.m. (seleniferous wheat)	10 p.p.m. as Na_2HAsO_3 in diet.
6	14 p.p.m. (seleniferous wheat)	10 p.p.m. as AsS_2 in diet.
7	14 p.p.m. (seleniferous wheat)	10 p.p.m. as AsS_3 in diet.
8	18 p.p.m. (seleniferous wheat)	None
9	18 p.p.m. (seleniferous wheat)	10 p.p.m. as Na_2HAsO_3 in drinking water.

of arsenic, as the same compound, when fed in the drinking water (group 3). The level of arsenic intake with a diet containing 10 p.p.m. of arsenic is slightly higher than the level of intake when rats are given 5 p.p.m. of arsenic in the drinking water which will account for the greater protection offered by the arsenic in group 5 than in group 3.

The arsenic sulfides (AsS_2 and AsS_3) used in groups 6 and 7 failed to give any indication of protection against the selenium in the diet. The mortality rate in these two groups was equal to that in group 2 where no arsenic was fed with the selenium.

As a continuation of the study, "the influence of arsenic and certain other elements on the toxicity of seleniferous grains" (Moxon and DuBois, '39), lead as lead acetate and bismuth as bismuth subnitrate were fed at levels of 5 p.p.m.
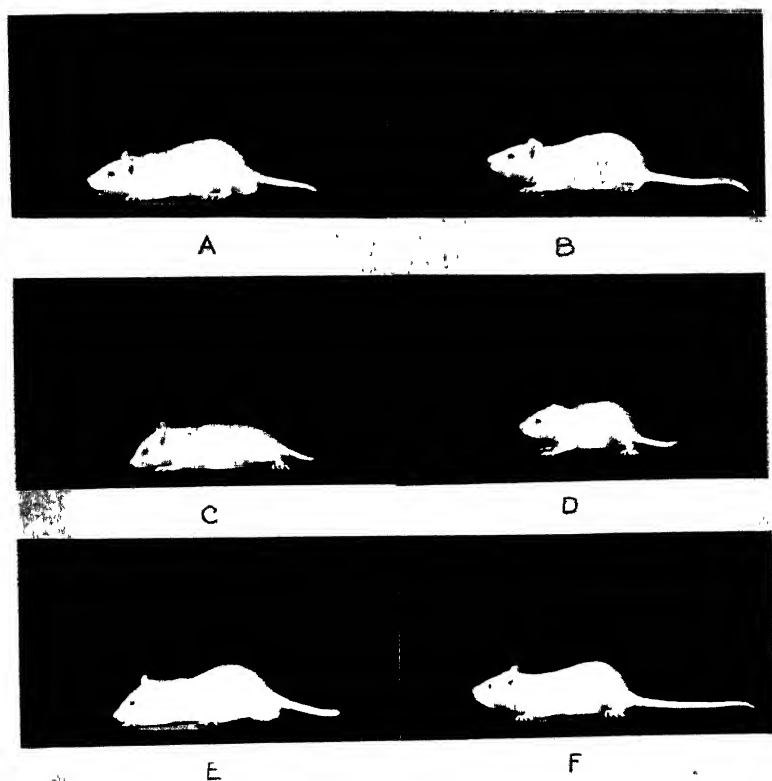
Fig. 1   A, C and E, males; B, D and F, females. A and B, control diet; C and D, seleniferous diet (18 p.p.m. Se); E and F, seleniferous diet (18 p.p.m. Se) + 10 p.p.m. arsenic in drinking water.

in the drinking water to rats on a diet containing 14 p.p.m. of selenium. In these experiments the lead did not influence the toxicity of the selenium during the duration of the feeding trials (100 days) but bismuth had a slightly beneficial effect.

Different forms of selenium

In a later experiment the efficacy of arsenic as Na_2HAsO_3 in preventing the toxic action of different forms of selenium was determined. The diet used was the same as in the above experiments. Selenium and arsenic were fed as shown in table 2.

TABLE 2

Effectiveness of arsenic as sodium arsenite against selenium in wheat and in sodium selenite

GROUP OF RATS	SELENIUM USED (IN DIET)	ARSENIC USED (IN DRINKING WATER)
1	None	None
2	11 p.p.m. Se (seleniferous wheat)	None
3	11 p.p.m. Se (seleniferous wheat)	5 p.p.m. As
4	11 p.p.m. Se (Na_2SeO_3)	None
5	11 p.p.m. Se (Na_2SeO_4)	5 p.p.m. As

The rats in group 2 were all dead within 35 days while those in group 4 were all dead at the end of 60 days. All rats in groups 3 and 5 were still alive at the end of 100 days when the experiment was terminated. The growth curves and condition of the livers on autopsy indicated that the arsenic as arsenite (Na_2HAsO_3) gave full protection against the toxic action of selenium as seleniferous wheat and as sodium selenite.

In studies involving the comparative toxicities of selenium as selenium-cystine, sodium selenite and seleniferous wheat[2] we have found that 10 p.p.m. of arsenic as arsenite (Na_2HAsO_3) will give adequate protection against 18 p.p.m. of selenium as selenium-cystine.

The response to arsenic after selenium had been fed for various periods of time

Soon after it was found that arsenic would protect against selenium poisoning (Moxon, '38) we became interested in determining what effect arsenic would have on animals in

[2] Unpublished data, this laboratory. 1938.

various stages of selenium poisoning. Six groups of rats were used as indicated in table 3.

The results indicate that arsenic is quite effective if selenium has been fed less than 30 days or not more than 20 days before the arsenic treatment is initiated. When selenium had been fed for 30 days before arsenic feeding began there was little growth response (compare groups 2 and 6 in the last column of table 3.

Liver glycogen levels have been determined (Potter, DuBois and Moxon, '39) on rats which had been fed selenium and selenium plus arsenic. Rats which had been fed a selenium diet had a lower glycogen level than the controls or the selenium plus arsenic groups.

TABLE 3

The response to arsenic after selenium has been fed for varying periods of time

GROUP OF RATS	DIET	ARSENIC (IN DRINKING WATER)	AVERAGE WEIGHTS AT	
			30 DAYS	90 DAYS
1	Control (no selenium)	None	115	190
2	18 p.p.m. selenium (wheat)	None	91	128
3	18 p.p.m. selenium (wheat)	10 p.p.m. As from beginning	109	187
4	18 p.p.m. selenium (wheat)	10 p.p.m. As after first 10 days	105	183
5	18 p.p.m. selenium (wheat)	10 p.p.m. As after first 20 days	102	174
6	18 p.p.m. selenium (wheat)	10 p.p.m. As after first 30 days	88	131

The protein content of diets used in selenium studies is of extreme importance. It has been shown (Moxon, '37; Smith, '39) that high protein diets offer more protection against selenium poisoning than low protein rations. The same basal diet has been used for all of our selenium-arsenic studies on rats. It contains approximately 22% protein (N × 6.25).

Although some interesting clues are being followed, no explanation for the antagonistic action of selenium and arsenic can be offered at present. A similar antagonistic effect between elements has been reported for thallium and iodine.[3]

[3] Research item, Iodides as antidotes in thallium poisoning, Nature, vol. 142, p. 440. 1938.

SUMMARY

Sodium arsenite and sodium arsenate were equally effective as sources of arsenic for preventing the toxic action of the selenium present in seleniferous wheat in the diet of albino rats; the arsenic sulfides (AsS_2 and AsS_3) were ineffective.

Arsenic as sodium arsenite was equally effective against selenium when the latter was given as seleniferous wheat, sodium selenite and selenium-cystine.

Arsenic was effective in treating rats which had been fed selenium for 20 days but was of little value after selenium had been fed for 30 days.

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NICOTINIC ACID POTENCY OF FOOD MATERIALS AND CERTAIN CHEMICAL COMPOUNDS [1]

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ONE FIGURE

The extensive use of nicotinic acid in the treatment of human pellagra (Spies, Bean and Ashe, '39) renders a knowledge of its quantitative distribution in various food materials of considerable interest. A number of assay methods, both chemical and biological, have been worked out, but quantitative data have been made available only through use of the chemical methods.

The chemical methods available at present for the determination of nicotinic acid in biological materials give varied results. Colorimetric methods have been reported by Swaminathan ('38), Karrer and Keller ('39), Kringstad and Naess ('39), Bandier ('39) and Harris and Raymond ('39). The greatest difficulty encountered is the complete extraction of the active compounds from the materials investigated. Since little information is available on the actual form of nicotinic acid in naturally occurring materials, the best method of extraction of this compound must await further investigation. The colorimetric determinations are further beset by the disadvantage that they require a colorless extract for the most convenient development of color. Any effort to adsorb

extraneous pigment from an extract may bring about the adsorption of nicotinic acid as well, thus giving low values.

The biological method depends on the treatment or prevention of the specific disease black tongue in dogs. Using the black tongue preventative technique Goldberger et al. ('28) and Sebrell et al. ('34, '35) were able to determine qualitatively the distribution of the anti-pellagra factor in a number of foodstuffs. The use of dogs for bio-assay purposes has the advantage of furnishing information as to the actual nicotinic acid potency of a particular food, regardless of the form in which nicotinic acid occurs. During the past 2 years we have used this bio-assay for determining the nicotinic acid potency of (1) meats and meat products, (2) other food materials, and (3) certain specific chemical compounds related to nicotinic acid.

Mongrel dogs were used in all of the assays.[2] They were placed on a modification of the Goldberger ration (Goldberger, Wheeler, Lillie and Rogers, '28) having the following composition:

	%
Ground yellow corn	71
Purified casein [3]	18
Salts [4]	4
Cottonseed oil	5
Cod liver oil	2

Since similar rations have been shown to be deficient in riboflavin and thiamin (Helmer and Fouts, '38; Margolis, Margolis and Smith, '39) in addition to nicotinic acid, these two pure vitamins were given weekly in aqueous alcoholic solutions at a level of 50 mcg. per kilogram of body weight per day.[5] When it appeared likely that corn contained less of vitamin B_6 than was previously supposed, vitamin B_6 [6] was also given twice weekly in dosages of 500 mcg. per dog.

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[2] We are indebted to the Works Progress Administration project no. 8649 for assistance in the care of animals.

[3] Washed eight times, reprecipitated twice.

[4] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935) salt mixture with $MnSO_4 \cdot 4H_2O$ increased from 0.7 to 10 gm. per kilogram of salt mixture.

[5] [6] We are indebted to Merck and Company for generous supplies of thiamin, nicotinic acid and vitamin B_6.

The dogs developed typical black tongue in from 1 to 3 months. When the first distinct drop in weight was observed, a dog was given a standardizing dose of 20 mg. of nicotinic acid by capsule. The animals responded to the vitamin within 12 to 24 hours by beginning to eat the ration. Usually in 5 to 14 days the restoration of weight had reached its peak, and in 14 to 21 days the dogs had lost weight and reached the level at which they were standardized. The response in weight to the standard dosage of nicotinic acid was noted and the gain in weight per milligram of nicotinic acid was calculated.
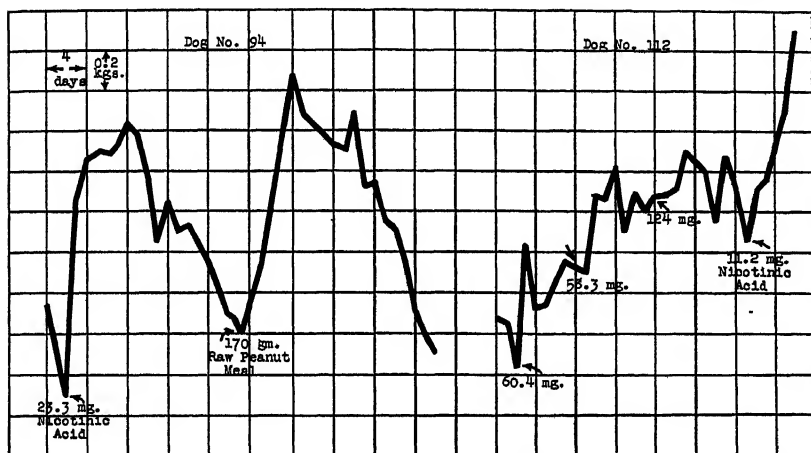
Fig. 1   Curve 1. Standardization with nicotinic acid followed by an assay of peanut meal. Curve 2. Effect of 3 doses of pyrazine monocarboxylic acid followed by one of nicotinic acid on the growth of a young pup.

A foodstuff to be assayed was then fed to the dog at a level which would contain approximately the same amount of nicotinic acid as the original standardizing dose. The weight response elicited by the supplement was noted and by a simple proportion the amount of nicotinic acid per gram of material was calculated. A typical response to a standard amount of nicotinic acid followed by an assay of peanut meal is shown in figure 1.

Repeated attacks of black tongue in a dog may decrease the animal's efficiency for assay purposes due possibly to gastro-

intestinal lesions. Some indication of the fact that certain dogs have a decreasing ability to respond to a supplement was obtained in a few animals and the assays in these cases were discarded. In order to minimize any error each animal was restandardized after two successive assays by again giving 20 mg. of nicotinic acid by capsule to the dog. To further increase the accuracy of the assays a foodstuff was usually fed to more than one dog or was reassayed on the same dog at a later period.

<center>RESULTS</center>

The values obtained for meats and meat products are given in table 1. The meat samples tested were those used in previous assays for thiamin (Mickelsen, Waisman and Elvehjem, '39), riboflavin (Mickelsen, Waisman and Elvehjem, '39), and chick antidermatitis factor (pantothenic acid) (Waisman, Mickelsen and Elvehjem, '39). Each sample of dried meat was fed in three equal portions suspended in a small amount of water when the dog had reached the weight at which it was standardized.

The colorimetric determination of nicotinic acid in beef muscle gives approximately 4 to 5 mg. per cent (Bandier, '39; Kringstad and Naess, '39; Karrer and Keller, '39), while we obtained 3.8 in one sample and 10.2 in another sample of the same tissue. Bandier ('39) and Kringstad and Naess ('39) found 4.7 and 3.3 mg. per cent respectively in pork muscle, while our values ranged from 5.3 to 13.0 mg. per cent. The beef spleen analyzed by Bandier ('39) was slightly lower than the range of nicotinic acid found in our samples of the same tissue. The values for pork heart and beef heart found by Bandier check within the range found in our samples. Whereas Bandier ('39) found 6.5 mg. per cent for beef kidney and 6.8 for pork kidney, Karrer and Keller ('39) found 19.4 for beef kidney which agrees well with 16.9 found in our sample of beef kidney, and with 15.5 mg. found in our sample of pork kidney. Kringstad and Naess ('39) found 18.0 mg., Bandier ('39) found 12.2 mg., and Karrer and

TABLE 1

Nicotinic acid content of meats and meat products

SAMPLE	SAMPLE NUMBER	MILLIGRAMS OF NICOTINIC ACID PER 100 GM. OF THE FOOD		
		Dry	Fresh	Fresh, as reported in the literature
Pork liver	33	90.0	26.4	11.8 (Bandier, '39)
Pork liver	122	110.0	27.5	
Beef liver	110	110.0	27.5	9.3 (Karrer and Keller, '39)
Beef liver	120	85.0	25.0	12.2 (Bandier, '39)
Beef liver, fried	121	87.0	29.4	
Veal liver	70	72.0	22.5	18.0 (Kringstad and Naess, '39)
Lamb liver	61	131.0	46.0	
Lamb liver	96	135.0	39.2	
Pork kidney	62	72.0	15.5	6.83 (Bandier, '39)
Beef kidney	84	81.0	16.9	6.5 (Bandier, '39)
				19.4 (Karrer and Keller, '39)
Beef kidney	126	89.0	17.8	
Pork heart	71	32.0	8.0	5.34 (Bandier, '39)
Beef heart	73	23.0	4.9	5.93 (Bandier, '39)
Beef spleen	76	28.0	7.0	4.42 (Bandier, '39)
Beef spleen	108	33.0	8.3	
Beef spleen	123	52.0	12.3	
Pork ham	95	40.0	9.7	4.73 (Bandier, '39)
Pork ham	32	38.0	10.4	3.3 (Kringstad and Naess, '39)
Pork ham	114	37.0	10.0	
Pork ham	118	32.0	8.8	
Boiled ham	115	15.0	5.2	
Smoked ham	116	28.0	8.2	
Tenderized ham	119	25.0	8.3	
Pork loin	74	46.0	13.0	
Pork loin	92	25.0	7.5	
Pork loin	125	19.0	5.3	
Beef tongue	82	46.0	12.8	
Veal hindquarter	75	70.0	16.1	
Veal hindquarter	44	72.0	18.0	
Veal hindquarter	103	24.0	6.5	
Beef brain	77	30.0	7.5	
Beef muscle	40	15.0	3.8	4.1 (Bandier, '39)
Beef muscle	105	37.0	10.2	3.8 (Karrer and Keller, '39)
				4.9 (Kringstad and Naess, '39)
Roast beef	107	37.0	10.2	
Beef pancreas	79	11.0	2.7	
Beef pancreas	64	16.0	3.5	
Beef lung	78	33.0	8.3	

Keller ('39) found 9.3 mg. in their samples, of beef liver while our values were somewhat higher. Our assays show that pork liver contains about the same as beef liver. Bandier ('39) found less than half of our values in these tissues.

It will be seen by inspection of the data that the organ tissues are richer in anti-black tongue activity than muscle tissue. This has been a consistent finding in the previous assays reported from this laboratory on the distribution of the various factors of the vitamin B complex in meats and meat products. Recently, work by Axelrod, Madden and Elvehjem ('39) showed that both liver and kidney of the dog and rat contained more coenzyme I (cozymase, nicotinic acid contain-. ing coenzyme) than the muscle and brain tissues of these animals. Their work demonstrated that pig kidney and brain maintained their normal coenzyme I content but decreases were always noted in the liver and muscle in nicotinic acid deficiency. Although no positive correlations could be made from their work, there was an indication that the coenzyme I content was a function of the degree of the deficiency. The tissues used for assay may then be expected to vary in their nicotinic acid content depending upon the state of nutrition of the animal.

In table 2 the values for food materials other than meats are listed. The values for yeast obtained with this method compare favorably with those reported by Swaminathan ('38), Bandier and Hald ('39), and Kringstad and Naess ('39) who used chemical methods, but the high variation in nicotinic acid content of different yeast samples makes such a comparison difficult. The results obtained with liver extract are consistently higher than those obtained by chemical methods. Shaw and MacDonald ('38) reported a maximum of 49 mg. per cent of nicotinic acid for liver extract powder using the cyanogen bromide method, while we have obtained the value of 270 mg. per cent. Liquid liver extracts vary greatly in their method of preparation and in total solids content. Our high value of 450 mg. per cent was obtained from an alcohol soluble

liver extract representing a high concentration from fresh liver.

The value obtained for skim milk powder, however, was only approximately one-half that reported by Swaminathan ('38), while our value for unroasted peanut meal reduced to terms of his ether extracted meal compares favorably with his assay. The very low activity of eggs, wheat germ, and extracted wheat germ is of interest since these had been reported

TABLE 2

FOODSTUFF OR SOURCE MATERIAL	NICOTINIC ACID mg.%
1. Skim milk powder	4.3–6.2
2. Bakers yeast	50
3. Brewers yeast Sample A	93
B	90
C	35
D	34
E	39
F	56
4. Liver extract, alcohol soluble (Wilson Laboratories no. 36997)	450
5. Liver fraction ''B'' (Wilson Laboratories no. 38818)	210
6. Liver extract powder (Wilson Laboratories no. 38785)	270
7. Alcohol-ether precipitate fraction of liver extract powder	less than 35
8. Rice bran extract, a commercial	165
9. Whey concentrate, a commercial	less than 15
10. Peanut meal (raw)	13
11. Grass, a commercial preparation	7.8
12. Egg yolk (hard boiled)	less than 4
13. Egg white (hard boiled)	less than 2.5
14. Wheat germ	less than 4
15. Extracted wheat germ, a commercial	less than 6

to be fairly good sources of the factor by Goldberger et al. ('28) and their inclusion in pellagra curative diets was recommended.

The effective use of quinolinic acid in the treatment of human pellagra as reported by Vilter and Spies ('39) suggested another trial of this compound in the treatment of acute black tongue. Since a previous assay had failed orally (Woolley, Strong, Madden and Elvehjem, '38) a dose of 213

mg. was injected.[7] This produced no effect. It is therefore
apparent that quinolinic acid is decarboxylated neither in the
dog intestine nor by the tissues, and that it cannot be utilized
as such by the tissues. The point of decarboxylation of
quinolinic acid in the human body is still unknown since it
was administered only by the oral route.

The efficacy of pyrazine mono- and 2, 3 dicarboxylic acids
as reported by Bills, McDonald and Spies ('39) when ad-
ministered orally to human pellagrins suggested a trial of
these compounds. Pyrazine monocarboxylic acid was in-
effective in restoring weight to or even maintaining dogs thus
depleted when given orally at a level of 106 mg.; pyrazine 2, 3
dicarboxylic acid was ineffective at a level of 104 mg. and
again at 195 mg.[8] All these levels were at least five times
the magnitude of the standardizing dose of nicotinic acid.
However, slight responses obtained with growing puppies on
this ration (fig. 1) with both compounds might indicate a
mobilizing effect of these compounds for nicotinic acid.

The growth stimulating effect of thiazole-5-carboxylic acid
for dysentery bacilli when substituted for nicotinic acid in
the basal synthetic media as reported by Schmelkes ('39)
suggested an assay of this compound. Both the acid and its
amide were inactive at levels of 310 and 440 mg. respectively,
although another assay of the amide using 1.05 gm. indicated
slight activity.[9]

<div align="center">DISCUSSION</div>

It has been shown that the more recent results obtained
by improved chemical methods are not greatly different from
the bio-assays. In general the bio-assay gives higher figures
which may be due to the fact that the animal organism will
efficiently utilize all forms of nicotinic acid derivatives. While
the chemical method may be limited in its reaction, it is also

[7] Furnished by Prof. S. M. McElvain of the Chemistry Department, University
of Wisconsin.

[8] Furnished by Dr. Tom D. Spies.

[9] Furnished by Dr. F. C. Schmelkes of Wallace and Tiernan Company, New York.

likely that the response obtained by feeding foods such as liver, which contains practically all of the B complex vitamins, is slightly greater than the administration of nicotinic acid alone due to a more efficient utilization of the vitamin. If this is true the bio-assay may tend to give higher values than the chemical method. In any case the results presented in this paper give a relative indication of the anti-pellagra activity of the products tested.

SUMMARY

1. The biological assay for the nicotinic acid content of various food materials has been accomplished by the use of black tongue dogs maintained on the modified Goldberger diet.

2. The nicotinic acid content of meats and meat products ranges from 10 to 110 mg. per 100 gm. of dry tissue.

3. Liver extracts contained from 200 to 450 mg. of nicotinic acid per 100 gm. Yeast ranged from 30 to 100 mg. per 100 gm. dry weight.

4. Quinolinic acid was found to be inactive as a substitute for nicotinic acid when injected. Pyrazine mono- and 2, 3 dicarboxylic acid and thiazole 5 carboxylic acid were either inactive or of very low activity.

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PSEUDO-HYPOPHYSECTOMY

A CONDITION RESEMBLING HYPOPHYSECTOMY PRODUCED
BY MALNUTRITION

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Many dietary procedures which are intended to elucidate endocrine and other physiological functions of the rat result in decreased food intake and loss in body weight. We have reinvestigated the effects of starvation and of underfeeding upon the tissues of the rat. In addition to the anestrus and sterility which developed during malnutrition, the anatomic changes observed bore a close resemblance to those following hypophysectomy. Since this operation results in a decrease in food intake and a loss in body weight, it is possible that the concomitant inanition which follows ablation of the pituitary gland may be responsible in part for some of these tissue changes. On the other hand, Jackson ('25), from his studies on inanition, suggested that ". . . . changes produced in the hypophysis may be responsible for some of the general phenomena of inanition and malnutrition." Mason and Wolfe ('30) stated that, "The hypophysis of male rats subjected to chronic inanition showed a significant decrease in activity . . . ." (See also Marrian and Parkes, '29; Moore and Samuels, '31; Werner, '39.)

The problem presented is to determine to what extent the effects of inanition on the rat are due to the primary tissue malnutrition and to what extent they are secondary to the

diminished elaboration of hormones by the malnourished endocrine glands. It is the purpose of this report to elucidate the part played by the hypophysis during inanition. Changes produced in the pituitary have been found to be at least in part responsible for many of the tissue changes occurring in inanition. The effects of inanition upon certain tissues we have termed pseudo-hypophysectomy (Pomerantz and Mulinos, '39) in order to stress their resemblance to true hypophysectomy and to distinguish them from the tissue changes due directly to the malnutrition.

<div align="center">PROCEDURE</div>

Approximately 300 albino adult rats were employed for this study. Each procedure was carried out on groups of five rats of the same sex which were kept in a single cage. The food and water intake of the group was determined daily together with the body weight of each rat. Daily estrous smears were taken and also palpation for size and position of testes was made.

The rats were subjected to two types of inanition:

1. Complete inanition, induced by withholding all food and water. Two-hundred-gram rats lived approximately 10 days (7–13) under these conditions. Tissue changes were most marked just before death; they were greatest in those rats which lost the greatest percentage of body weight.

2. Chronic inanition, induced by allowing one-half of the amount of the complete food mixture [1] that the rats had been eating during a preliminary period of observation. Each rat was allowed from 6 to 8 gm. of food per day, approximately the same as that reported to be ingested voluntarily by hypophysectomized adult rats (Lee, '38).

Control groups of rats fed the full diet were always run in parallel with the inanition experiments. In this way, seasonal,

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[1] Egg—Breeder Mash, Quaker Oats Co. Chemical analysis: protein 20%; fat 4.5%; fiber 8%, carbohydrates (nitrogen-free extract) 45%. Ingredients: oatmeal, hominy feed, wheat bran and middlings, barley feed, alfalfa meal, cereal grass, fish meal, meat scraps, cod liver meal, sardine oil, dried whey and buttermilk, cane molasses and 0.75% salt.

room temperature, weight, sex, and age influences were reduced to a minimum.

At the predetermined time in each experiment, the rats were anesthetized lightly with ether, the chest wall opened, the animals bled to death from the heart, and autopsy was performed immediately. In most of the autopsies the hypophysis, thyroids, thymus, spleen, liver, kidneys, adrenals, and sex organs were carefully dissected, and weighed individually on an analytical balance.

RESULTS

Since the effects of inanition in the rat were found to be essentially the same as those described by numerous investigators and thoroughly reviewed by Jackson ('25), this report concerns itself with a comparison between inanition and hypophysectomy and not with a complete description of these conditions. The evidence presented reveals that the tissues of adult underfed rats anatomically and physiologically resemble in many respects those of adult hypophysectomized rats as reported in the literature.

The adrenal glands. After about a week of complete inanition, the adrenal glands may be larger and heavier than in fed controls (fig. 1). They may be congested with blood and red in color. These changes resemble those reported by Perla ('35) to occur within the first 2 weeks after ablation of the hypophysis. Chronic inanition, however, resulted in atrophy of the adrenal cortex (fig. 1). The atrophy was never marked, and involved chiefly the cytoplasm, increasing the nucleus-plasma ratio. The findings are similar to those of Jackson ('25). These cellular changes resemble those resulting from hypophysectomy in which, "The cells of all three zones exhibit a marked diminution in their cytoplasm. The nuclei are consequently massed together. It appears that the reduction of the cortex is not affected through disappearance of cells, but rather by this diminution in their cytoplasmic content" (Smith, '30 a).

During inanition, the cytoplasm of the cells of the cortex and especially of the zona reticularis is diminished, thereby

making the nuclei appear relatively numerous, while the dark-staining chromatin is arranged in spoke-like fashion (fig. 2). Cells of this type found in the ovary have been designated "wheel cells," because of the nuclear appearance (Selye, Collip and Thomson, '33). They have not been reported as
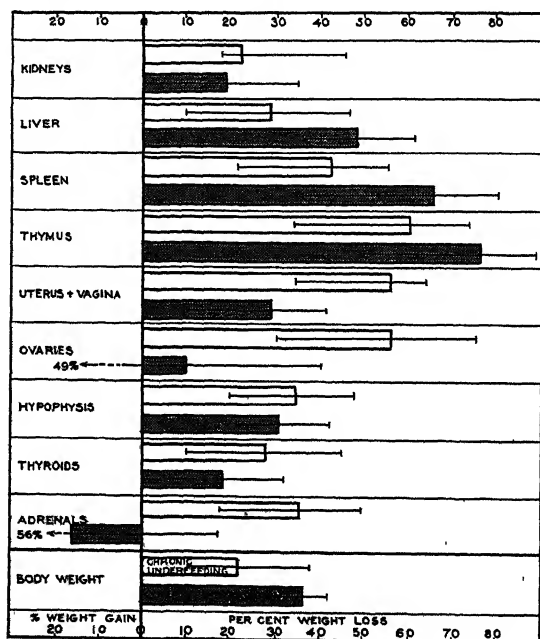
Fig. 1   Relationship between the loss in body weight and the loss in organ weight during complete (stippled bars) and chronic inanition. The chart is based upon data from a representative group of twenty normal control, thirty completely starved (5 to 11 days), and twenty-five chronically underfed (26 to 182 days) female rats. The average initial body weight was 200 gm.

The bars indicate the average percentage weight change from the normal controls, while the thin lines within the bars represent the range of variation.

occurring in the adrenal cortex of the hypophysectomized or underfed rat.

Chronic inanition in the rat is followed by deposition of a greenish-yellow pigment in the zona reticularis of the adrenal cortex and is especially marked in rats which are underfed for long periods (3 to 4 months). The pigment masses vary in size from fine particles to particles larger than the neighboring

nuclei. These pigmentary deposits are similar to those described by Cutuly and Cutuly ('37) as occurring in the adrenals of hypophysectomized rats. They believed that the pigment "represented a type of degeneration resulting after ablation of the anterior lobe of the pituitary."
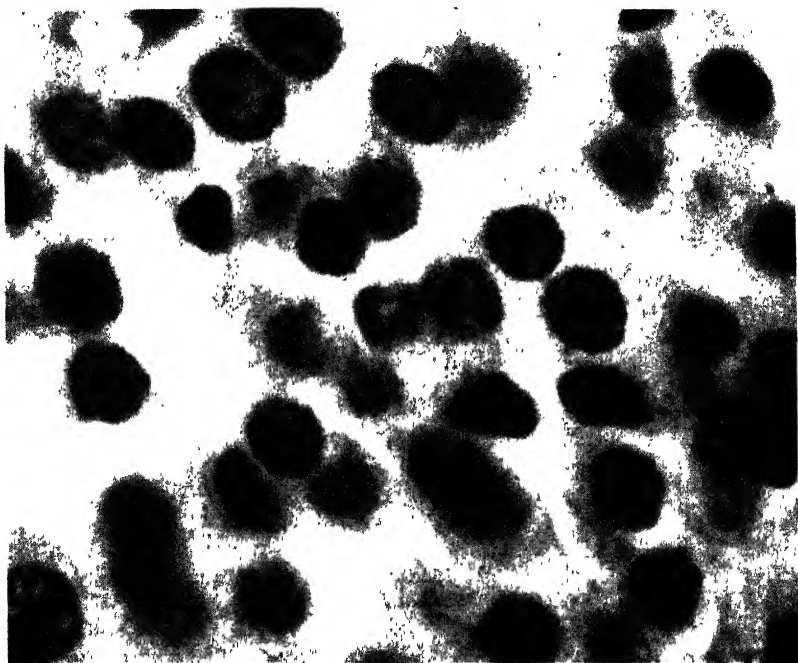
Fig. 2   Wheel cells in the cortex of the adrenal gland of a rat underfed for 42 days. Note the spoke-like arrangement of the nuclear chromatin. The animal lost 17% of its body weight and the adrenals weighed 64% (30 mg.) of the average normal adrenal weight (47.4 mg.). Similar cells appear in ovaries and interstitial tissue of the testes of chronically underfed rats.

The adrenal medulla is virtually unaffected either after hypophysectomy or during chronic inanition. It may appear relatively enlarged because of the cortical atrophy. The medullary cells retain their chromaffin reaction in both conditions.

The thyroid gland. In complete inanition the loss in the weight of the thyroid gland is relatively less than the loss

in body weight, whereas in chronic inanition it is greater (fig. 1). The atrophy from inanition is never so great as after hypophysectomy. The histologic changes are qualitatively similar, however. The cuboidal cells of the follicles are atrophied and reduced in height. The nuclei stain more deeply, and although shrunken, they appear relatively more prominent. The intrafollicular colloid persists even until the death of the rat.

The spleen. In both total inanition and chronic inanition with continued weight loss there is a decrease in the weight of the spleen proportionately greater than the loss of body weight. The atrophy is most marked under complete inanition when the weight loss may be over 60% (fig. 1). There is atrophy of the lymphoid tissue. The malpighian corpuscles are reduced in size and appear relatively numerous.

After hypophysectomy there is a similar reduction in the size of the spleen (Smith, '30 a). Perla ('36) found that 2 months after hypophysectomy the ratio of spleen to body weight fell to about one-half the normal. There was a decrease in the size of the follicles of these spleens with an increase in their number per unit area. Deposits of a greenish-yellow pigment (giving the Prussian-blue reaction) which are found in spleens of hypophysectomized rats (Kutz, McKeown and Selye, '34) also occur in the spleens of our starved or underfed rats.

The liver. In our rats, complete and chronic inanition caused a relatively greater loss in liver weight than in body weight (fig. 1). Some of the liver weight loss is due to the extreme glycogen depletion which is a part of the rapid diminution in all carbohydrate stores, and is more apparent the greater the inanition.

After hypophysectomy there is a loss of liver weight which is usually relatively greater than the loss in body weight (Smith, '30 a). Franseen, Brues and Richards ('38), and Higgins and Ingle ('39) independently noted that after partial hepatectomy the rate of liver restoration was greater in control (fully fed) than in hypophysectomized rats. However, these investigators reported that when control rats were

underfed purposely and made to lose weight parallel to the hypophysectomized animals, the rate of liver restoration was essentially equal in both groups.

The thymus. Complete and chronic inanition in our rats resulted in marked involution of the thymus gland, the percentage loss in weight being far in excess of the body weight loss (fig. 1). There was a decrease of the lymphoid elements with an apparent increase in connective tissue. In extreme cases of inanition the clear differentiation between the cortex and medulla disappeared.

According to the data presented by Smith ('30 b), the loss of thymus weight after hypophysectomy in the rat was also relatively greater than the loss of body weight. Smith ('30 b) considered the possibility that inanition may have contributed to the thymus weight changes.

Hair growth. Small areas of skin on the backs of chronically underfed and normal control rats were depilated with a barium sulfide mixture. Inanition now delayed the regrowth of hair from a few days to several months beyond the normal period; the retardation was roughly proportional to the degree of inanition. Following hypophysectomy, the growth of hair over shaved areas of skin has been reported to be slower than in unoperated fully fed controls (Snow and Whitehead, '35).

The female reproductive system. Anestrus usually occurs in adult rats during inanition (Evans and Bishop, '22) and often develops when body weight loss is only 15% (Mulinos et al., '39). Hypophysectomy is also followed by anestrus. Coincident with the loss of estrual cycles resulting from complete inanition, the number and size of the graafian follicles are decreased, yet the weight and size of the ovary are not markedly altered (fig. 1). Within 3 weeks chronic inanition usually results in changes in the structure of the ovary which are qualitatively similar to those reported to occur after hypophysectomy. Primordial follicles continued to develop throughout the period of inanition, but upon reaching the stage of antrum formation, they become atretic. Within 3 weeks the ovary has shrunken in size and contains many

atretic follicles. During this time, when there is usually a typical anestrous vaginal smear, the ovaries contain but a few degenerated corpora lutea, in contrast to the persisting corpora lutea of hypophysectomy. After 2 months of chronic inanition during which there has been no typical estrual activity, it is not unusual to find a few nucleated and cornified epithelial cells in a vaginal smear predominantly leucocytic. This is especially true in the rats which have lost least in weight. The ovaries of these rats often contain several well-formed corpora lutea. The presence of corpora lutea after more than 2 months of anestrus is indicative of the development of some follicles into corpora lutea, but in number too few to yield sufficient estrogenic hormone for the appearance of a typical estrous smear. The elaboration of estrogenic hormone by such ovaries is reflected by the fact that the adnexa of these animals are heavier than those in the completely anestrous rats.

Around the degenerated follicles of the ovary of the hypophysectomized rat there are thecal cells which are poor in cytoplasm and contain a "wheel-like" nucleus not unlike that of a plasma cell. These "wheel-cells" have been described by Selye et al. ('33) as being ". . . . a typical deficiency reaction of the ovary after hypophysectomy . . . ." Cells indistinguishable from these "wheel-cells" have been found by us to occur in the ovaries of rats during chronic inanition (Pomerantz and Mulinos, '39). The number of "wheel-cells" is related to the degree and duration of the inanition. If it be allowed that inanition decreases the hormonal activity of the anterior pituitary gland, a common etiology for the origin of the "wheel-cells" of hypophysectomy and chronic inanition becomes evident.

The loss in adnexal weight is progressive and as the period of inanition lengthens, the weight loss becomes relatively greater than the body weight loss (fig. 1). The greater the atrophy of the ovary from inanition, the greater is the atrophy of the adnexa. Pituitary substance (Marrian and Parkes, '29), gonadotropic hormone (pregnancy urine extract) or estrogen (Mulinos et al., '39) injected into chronically

underfed animals result in adnexal hypertrophy and the appearance of estrus. The adnexa of hypophysectomized rats respond in a similar manner to the injection of these hormones. An insufficiency of estrogen appears to be common both to inanition and to hypophysectomy and thus the cause of the anestrus and adnexal atrophy.

The male reproductive system. Complete inanition unto death results in the destruction of an occasional seminiferous tubule, whereas prolonged chronic inanition results in severe atrophy of the testes and accessory genitalia. After chronic inanition the changes in the testes are qualitatively similar to those described as occurring in the adult rat after hypophysectomy (Smith, '30 a). The atrophy is more severe in the latter but the results are complicated by the fact that the testes are pulled up into the abdomen soon after hypophysectomy. After 50 days of chronic inanition the testes and accessories may weigh less than one-fifth the normal despite the fact that the testes remain in the scrotum. The production of spermatozoa is abolished after hypophysectomy and diminished or absent during inanition. There is generally no destruction of the Sertoli cells or the spermatogonia in either condition. There appears to be no hypertrophy of the interstitial tissue concurrent with the tubular degeneration due either to inanition (Siperstein, '21) or to hypophysectomy (Smith, '30 a). Stained sections of the testes of rats with chronic inanition contain many interstitial cells with "wheel-like" nuclei which resemble those occurring in the adrenal cortex and the ovarian stroma. These cells have not been described in the atrophic testes of the hypophysectomized or the underfed rat.

The hypophysis. The hypophysis lost in weight relatively less during acute than during chronic inanition, the loss in the latter being relatively greater than the body weight loss. Histologically, the gland showed evidence of atrophy as already described by Jackson ('25). The atrophy of the hypophysis may result in an impairment of function. Werner ('39) has shown that the hypophyses of chronically underfed

rats contain less gonadotropic principles than those of normally fed rats.

DISCUSSION

The responses of the various organs of the body to severe and continued inanition may be divided into three groups or degrees:

Group I. The most obvious effects of inanition are those due to direct malnutrition from a deficiency in calories, minerals, vitamins, etc. In this first group probably belong the kidneys, and other structures, the loss of weight in which is usually proportional to the loss in body weight.

Group II. A secondary effect of inanition may be expected to affect organs which are dependent for their full activity upon hormones elaborated elsewhere. For example, the pituitary gland may respond to inanition by the primary reaction described under group I with a fall in the quantity and perhaps the quality of its secretions. This results in a diminution of the concentration of gonadotropic principle to the end that a primarily malnourished ovary will receive less stimulation than normally, and become secondarily depressed. The ovarian response to inanition may, therefore, be a loss in weight greater in proportion than the loss in body weight, a phenomenon which actually occurs. The testes and adrenals, and perhaps the thyroid and spleen, are some of the organs which may be secondarily affected by the primary effect of inanition upon the anterior pituitary gland.

Group III. Following the reasoning under group II, a tertiary effect may result from the secondary. An example is offered by the response to inanition of the sex accessory organs of male and female rats. The factors involved in this response would be threefold: (a) direct malnutrition of the accessories themselves, (b) diminution of the secretion of accessory-stimulating sex hormone by the poorly nourished gonads, and (c) further diminution of gonadal secretion due to the malnutrition of the pituitary gland, and the consequent fall in the secretion of gonadotropic hormone.

The tissue changes resulting from underfeeding are of the greatest importance in the consideration of data from experiments purporting to elucidate certain problems. In any experimental procedure which results in a loss of body weight one must take into account the effects of the concomitant inanition. As far as the rat is concerned malnutrition complicates all toxicological experiments, and many dietary ones, especially those in which vitamin B_1 is lacking. All such experiments must be run in parallel with controls which are made to lose in weight at the same rate as the experimental animals. Finally, it is essential to distinguish between acute and chronic inanition since the organ changes are often qualitatively dissimilar in the two conditions.

SUMMARY

Data have been obtained from over 300 rats upon acute and chronic inanition, and then compared with the tissue changes reported to occur after hypophysectomy. The effects of prolonged chronic inanition resemble those from hypophysectomy, especially upon the endocrine organs. The endocrine tissue changes are qualitatively similar in the two conditions but they are seldom so marked during chronic inanition as they are following hypophysectomy. Other tissue changes are equally marked in both conditions and appear to be related to the loss in the body weight.

The response of the endocrine organs of the rat to chronic inanition has been termed pseudo-hypophysectomy not only because of the resemblance between the effects of inanition and hypophysectomy but also because we believe that many of the effects of inanition are due to malnutrition of the hypophysis, resulting in a diminished secretion of hormones.

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PROTEIN ANABOLISM IN THE ORGANS AND TISSUES OF PREGNANT RATS AT DIFFERENT LEVELS OF PROTEIN CONSUMPTION [1]

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ONE FIGURE

This investigation is a sequence to a previous study on the distribution of protein in the body of the pregnant rat (Poo, Lew and Addis, '39). It was required because we made two observations we could not comprehend. One was the fact that the concentration of protein in the serum, carcass, liver, blood clot, kidney and heart was reduced relatively to non-pregnant controls. Since the rats had been maintained on a diet that did not contain much protein we were not sure whether this decreased protein concentration was an intrinsic characteristic of the pregnant state or was indicative of a relative protein deficiency arising because in pregnancy the rate of protein anabolism was more than double that of non-pregnant controls. The other observation was that the proportion of the total maternal protein assigned to the liver during pregnancy was much greater than in the controls. In this case two possible explanations occurred to us: this might be due to a work hypertrophy of the liver in pregnancy or, much less probably, to an accumulation of protein to be used later during lactation. By making similar measurements of protein distribution in pregnant rats kept on diets that varied widely in protein content one can determine whether this increased allocation of

505

protein to the liver is independent of or related to the supply of food protein. Under such conditions one can also answer the question as to the significance of the decrease in tissue protein concentration in pregnancy since at the higher levels of protein consumption there will be no possibility of an inadequate protein intake. Under these conditions further information will be obtained with respect to the extraordinary changes that occur during pregnancy in the internal distribution of protein within the body. It is as though the uterus and its contents became a parasite on the mother and deflected to itself protein normally apportioned to other parts. We had observed, for instance, that only 76% of the total protein in a pregnant rat was found in the carcass (mainly muscle, skin and skeleton) as compared with 86% in non-pregnant rats subsisting on the same 16% protein diet. It is of practical importance for the dietetics of pregnancy to determine whether such deflections from the usual assignment of protein to the various organs and tissues of the body may be diminished or abolished by increasing the supply of food protein.

A number of female rats about 90 days of age that had never borne litters were taken from a colony that had been reared on a diet containing 18% of protein. They were kept with males for 5 days and those that were pregnant were divided into four groups in such a manner that at the beginning of the experiment the average body weight of each group was 150 gm. and in each case the distribution of individual body weights was similar. On this zero day each group was given a diet that differed with respect to its protein content but was otherwise the same. The four diets contained respectively 11%, 16%, 27% and 43% of protein. This variation was accomplished by having each diet contain 60% by weight of a mixture of cornstarch and commercial casein that had a 79.2% protein content. The diet with the lowest protein concentration had 10% of casein, the next 16%, the next 30% and the highest 50% of casein, the remainder of the mixture being made up to 60% with cornstarch. In addition all the diets contained 15% of lard, 10% of sardine oil, 9% of dry yeast, 2% of dry

alfalfa and 4% of Osborne and Mendel's salt mixture. After 18 days on these diets, when pregnancy on the average had continued for 20.5 days and delivery was imminent, the four groups were anesthetized, exsanguinated, and the protein content of their organs and tissues was determined. In order to ascertain how much protein had been formed during these 18 days another group of twenty non-pregnant rats whose average body weight was 150 gm. was killed at 0 days, i.e., the day on which the various diets were first given to the pregnant groups, and the protein content of their organs and tissues was measured. The methods used were those described by Addis, Poo, Lew and Yuen ('36). The quantities found and the corresponding fresh weights are given in table 1.

The survey of the results in table 1 will be facilitated by reference to figure 1 in which the amounts of newly formed protein are expressed as percentages of the amounts found at 0 days.

The curves for total protein show how great are the changes in anabolism that can be induced by varying protein consumption. When only about a gram of food protein per day is taken, the total anabolism is even less than in non-pregnant controls, although with any adequate protein intake the quantity of new protein formed is much greater than in non-pregnant controls. In both pregnant and non-pregnant rats, however, the highest rate of anabolism occurs when about 2.5 gm. of protein are eaten and in both, anabolism is decreased when larger amounts are consumed.

What we have termed "maternal protein" in figure 1 is the total body protein minus the protein of the uterus and embryos. If pregnancy had no effect on the mother the pregnant and control curves should be superimposed; not only are they divergent but they cross one another, indicating that the nature of the effect is dependent on the amount of protein that is consumed. When the amount taken is small the mother's tissues lose protein, i.e., the relation between the mother and the uterus and its contents is one of host and parasite. When larger amounts are taken the mother's tissues construct more

TABLE 1

Fresh weight and protein content per rat of organs and tissues before and after 18 days of increasing protein consumption during pregnancy

CATEGORY OF INTEREST [1]	BEFORE PREGNANCY AT "ZERO DAYS" ON STOCK DIET	DURING PREGNANCY AFTER 18 DAYS SUBSISTENCE ON DIETS VARIED WITH RESPECT TO PROTEIN			
Protein content of diet in per cent	18	11	16	27	43
Protein eaten per rat per day in gm.	..	1.04	1.95	2.89	4.44
Body weight—total in gm.	150.000	191.333	232.301	228.394	207.100
Body protein content—total in gm.	23.546	26.245	31.518	31.594	29.885
Maternal organism [2]					
Weight in gm.	150.000	166.203	194.476	195.742	176.620
Protein content in gm.	23.506	24.331	28.624	29.065	27.379
Young per mother					
Weight in gm.	......	18.090	27.057	24.380	22.231
Protein content in gm.	......	1.247	2.072	1.726	1.684
Kidney					
Weight in gm.	0.995	0.958	1.114	1.217	1.296
Protein content in gm.	0.161	0.153	0.177	0.193	0.205
Heart					
Weight in gm.	0.519	0.538	0.623	0.616	0.598
Protein content in gm.	0.083	0.089	0.103	0.103	0.098
Liver					
Weight in gm.	6.567	7.400	8.835	9.565	8.713
Protein content in gm.	1.256	1.307	1.602	1.838	1.727
Alimentary tract etc.					
Weight in gm.	25.751	30.959	32.209	35.060	29.552
Protein content in gm.	1.631	1.693	1.809	1.954	1.802
Blood clot					
Weight in gm.	2.452	2.235	2.545	2.875	2.551
Protein content in gm.	0.712	0.608	0.714	0.793	0.730
Blood serum					
Weight in gm.	2.876	3.213	3.450	3.520	3.160
Protein content in gm.	0.166	0.148	0.185	0.195	0.172
Uterus etc.					
Weight in gm.	0.290	7.040	10.768	8.272	8.249
Protein content in gm.	0.040	0.667	0.822	0.803	0.822
Carcass					
Weight in gm.	110.550	116.900	145.700	142.889	130.750
Protein content in gm.	19.497	20.333	24.034	23.989	22.645

[1] Definitions of organs and tissues used in this study, with the exception of that for "maternal organism protein," are given in Addis, Lee, Lew and Poo ('40).

[2] Total body weight or protein minus the weight or protein of uterus and embryos.

protein than the controls, i.e., the relation becomes one of a symbiosis in which the mother gains. The curves for carcass protein (mainly muscle, skin and skeleton) repeat the same story, but the loss on a low protein diet is greater and the gain with an adequate protein intake less.

The curves for organ protein, liver, kidney and heart show that each organ has its own individual mode of reaction to variation in protein intake. It is shown also that in the pregnant rat the liver has a relatively higher rate of anabolism than any other part of the strictly maternal body.

QUANTITIES OF NEW PROTEIN FORMED DURING 18 DAYS IN PREGNANT RATS, EXPRESSED AS PERCENTAGES OF THE AMOUNTS FOUND AT 0 DAYS
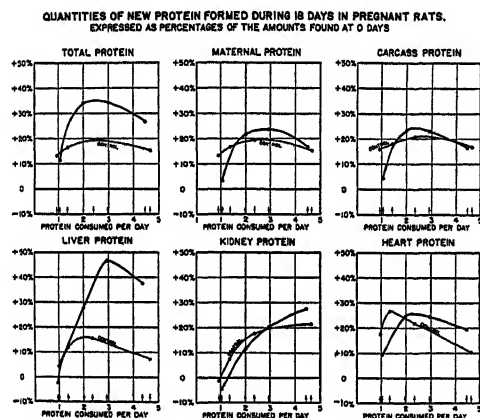
Fig. 1   The control curves are derived from data given in full in a recent number of this Journal (Addis, Lee, Lew and Poo, '40). These non-pregnant groups had the same initial body weights and were kept for 18 days on the same diets as the pregnant groups. The heavier lines are derived from the results in pregnant rats and were calculated from the data in table 1 of this paper.

The concentration of protein in the organs and tissues of pregnant rats (protein per 100 gm. organ or tissue weight) can be deduced from table 1. However, it is not the concentrations themselves but the question as to whether they are lower than the concentrations in the control non-pregnant groups that is of immediate interest to us. This relation is given in table 2 where the concentrations in pregnancy are expressed as + or — deviations from the concentrations in the controls.

The results given in table 2 show that the low concentrations we found in our previous work on a 16% protein diet were not due to a relative deficiency in protein consumption since low concentrations are still found with an entirely adequate protein intake and occur even when such an excess of protein is taken that the rate of anabolism of both mother and embryos is inhibited. Though the concentrations fall to their lowest level on a diet deficient in protein, this is only an accentuation of what is a constant characteristic of the state of pregnancy under all conditions. It remains true, of course,

TABLE 2

Concentration of protein in pregnancy and in non-pregnant rats after estradiol relative to the concentration in non-pregnant controls

ORGAN	PREGNANT RATS				NON-PREGNANT ESTRADIOL-TREATED RATS
	Dietary protein in per cent				Dietary protein in per cent
	11%	16%	27%	43%	16%
Serum	—39%	—20%	—16%	—20%	+8%
Alimentary tract etc.	—20%	—7%	—4%	—9%	+18%
Total maternal body	—11%	—5%	—6%	—5%	+4%
Carcass	—6%	—4%	—4%	—5%	+4%
Blood clot	—3%	—3%	—5%	—7%	—11%
Liver	—4%	—5%	—3%	—4%	+8%
Kidney	—4%	±0%	—2%	—7%	—2%
Heart	±0%	—2%	+4%	—4%	±0%

that no precise meaning can be attached to a decrease in protein concentration in material that consists of fat, minerals and water as well as protein. Inasmuch as serum and blood clot contain little fat and the mineral content is nearly constant, it is likely that a decrease in protein concentrations means dilution with water. Since there is no apparent increase in visible fat in pregnant rats, an increase in water content may also be the reason for the low concentrations in other tissues and organs. In any case this is a singular phenomenon for we have not found it under a variety of conditions in which pronounced changes in metabolism were induced by diet, thyroidectomy or thyroxin administration (Addis, Karnofsky,

Lew and Poo, '38). It is natural, therefore, to look for some circumstance peculiar to the pregnant state. One of these is the presence in the blood stream of relatively high concentrations of estrogenic substances.

We attempted to duplicate this peculiarity of pregnancy by injecting estradiol dipropionate into non-pregnant rats. All the other conditions of our present experiments were observed. Twenty rats were selected whose average body weight was 150 gm. on the day on which the diet containing 16% of protein was first given. They were killed 18 days later after having received 0.05 mg. of estradiol intramuscularly every other day, the last injection being given on the fifteenth day. Though the animals seemed to be quite healthy, their growth rate was slowed so that on the eighteenth day the average body weight was only 156 gm. In the last column of table 2 the protein concentrations are given expressed as a percentage of the concentrations in the control group on the same diet. Instead of being lower they are on the whole higher, a difference probably due to the fact that the animals in the estradiol-injected group were obviously leaner than the controls. There is no reason to suppose that the material we injected was ineffective, for the uterus, after the removal of the fluid it contained, weighed more than twice as much as that of the controls. When a single injection was given Zuckerman, Palmer and Bourne ('39) found an increase in the water content of the skin, and Thorne and Engel ('38) a decrease in sodium chloride and water excretion. It is possible that in our experiment with repeated injections over a period of 15 days, an equilibrium in salt and water exchange had been reestablished. In any event this experiment gives no support for the hypothesis that the lowered protein concentrations in pregnancy are due to the increase in estrogenic substances. We do not suppose that this hypothesis is thereby invalidated. It is obvious that we did not reproduce the actual hormone conditions that exist in the pregnant rat.

The distribution of protein (organ protein per 100 gm. total protein) has none of the ambiguity involved in measurements

of protein concentration in the body. Taking the total protein as representing 100%, this measure is a statement of the percentage assignment of the total available quantity among the various organs and tissues of the body. These organs and tissues each make their own demands. The proportion of the total they actually receive may be taken as evidence of their relative importance to the total economy. In pregnancy the total demand for amino acids for protein synthesis is increased and a new element is introduced by reason of the special requirements of the enlarging uterus and embryos. In pregnant animals subjected to a variation in food protein supply from amounts barely sufficient for maintenance to quantities in excess of all needs, we must consider changes in

TABLE 3

Distribution of protein in non-pregnant and pregnant rats

	NON-PREGNANT CONTROLS ON DIETS WITH PROTEIN CONCEN- TRATIONS IN PER CENT				PREGNANT RATS ON DIETS WITH PROTEIN CONCEN- TRATIONS IN PER CENT			
	11%	16%	27%	43%	11%	16%	27%	43%
	%	%	%	%	%	%	%	%
Uterus and young	0.2	0.2	0.2	0.2	7.3	9.2	8.0	8.4
Carcass and blood	88.0	87.5	87.4	87.3	80.3	79.1	79.1	78.8
Maternal organs	11.8	12.3	12.4	12.5	12.4	11.7	12.9	12.8
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

the distribution of protein if we desire to comprehend the effect of these conditions on the intermediary metabolism of protein within the body. The principal results are given in table 3 with the data for the controls.

In table 3 are presented our data with respect to three divisions of the body, namely, the uterus and its contents, the carcass and blood, and the maternal organs. The latter division includes all the abdominal and pelvic organs except the uterus. When such a gross division is made the results are clear. The proportion of protein allocated to the maternal organs remains substantially constant whether the animal is or is not pregnant and no matter what quantity of protein is consumed. The increase in the proportion of the total protein

that is allocated to the uterus and young is taken from the carcass and blood. Toward the end of pregnancy the amount of protein distributed among muscle, skin, skeleton and blood is diminished, but only relatively, for, as figure 1 shows, with an optimal supply of food protein the carcass protein is absolutely increased.

These are the main outlines of the effect of pregnancy on the distribution of body protein but there are subsidiary changes concealed by this method of presentation. Figure 1 shows that the absolute amount of protein laid down in the liver of the pregnant rat is out of proportion to the quantities formed by the livers of non-pregnant rats and that the gain is relatively greater in the liver than in the kidney or heart.

TABLE 4

Distribution of maternal organ protein in pregnancy relative to the distribution in non-pregnant controls

ORGAN	DIETARY PROTEIN CONCENTRATION IN PER CENT			
	11%	16%	27%	43%
Liver	+ 3%	+ 5%	+ 8%	+12%
Kidney	— 6%	— 7%	—13%	— 7%
Heart	—11%	—10%	—12%	— 5%
Alimentary tract etc.	— 1%	— 2%	— 4%	— 9%

We have seen that the proportion of the total protein allocated to the maternal organs is relatively constant. Within this constant organ proportion there may be differences in distribution of protein to the various organs that together make up the whole organ quantity. If this is the case the differences will be revealed by taking the whole maternal organ protein as 100% and ascertaining how this total amount is apportioned. The organ distributions in pregnancy relative to the distributions in the controls are given in table 4.

Table 4 shows that at all levels of protein intake the proportion assigned to the liver is greater than in the controls in contrast to the other organs where it is less. This, then, is a constant peculiarity of pregnancy. This is true whether the proportion of protein in the liver is calculated on the basis of

the total protein, of the total maternal protein, or of the maternal organ protein, though the difference, of course, is less marked if the total protein is considered as the unit. It is a difference that becomes more pronounced as the quantity of protein consumed as food increases. We are inclined to regard the phenomenon as an example of a work hypertrophy of the liver during pregnancy though we have no idea as to the nature of this work beyond the supposition that it is connected with protein metabolism and perhaps particularly with protein anabolism.

SUMMARY

1. The total quantity of protein formed during pregnancy is dependent on the protein intake. At low levels the quantity newly formed is even less than that of non-pregnant controls but with increasing protein intake it rises high above the control values and after attaining a maximum decreases as unusually large amounts of protein are consumed. The protein added to the mother's body (total protein less the protein of the uterus and its contents) is considerably less than that of non-pregnant rats when the protein intake is reduced to a gram per day, but when 2 or 3 gm. are eaten daily, the body of the pregnant rat gains more protein than the bodies of controls. In the organs the main effect of pregnancy is a pronounced increase in the protein of the liver at moderate and high levels of protein consumption.

2. The concentration of protein in the organs and tissues of pregnant rats is lower than that in non-pregnant rats at all levels of protein consumption. An attempt to reproduce in non-pregnant rats part of the hormone conditions of the pregnant state by the injection of estradiol propionate failed to support the hypothesis that the lowered protein concentrations were due to an increase in estrogenic substances.

3. At the end of pregnancy the gross distribution of protein in the body (organ or tissue protein per 100 gm. total protein) is not appreciably influenced by the amount of protein that has been taken as food. Roughly 8% of the total protein is

assigned to the uterus and embryos, 80% to the carcass and blood and 12% to the internal organs. All of the protein of the uterus and its contents represents that deflected from the carcass and blood which, in controls, accounts for 88% of the total. The proportion allocated to the internal organs is no different from that found in non-pregnant rats.

4. Although the proportion of the total protein assigned to the internal organs is about the same as the proportion found in non-pregnant animals the distribution among the various organs is different. In pregnant rats the liver obtains more protein and the other organs less. The preponderance of liver protein increases with increase in protein consumption.

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FASTING CATABOLISM AND FOOD UTILIZATION OF CALCIUM-DEFICIENT RATS

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ONE FIGURE

INTRODUCTION

According to Kriss and Smith ('37), "Rats kept on a mineral deficient diet were less active and invariably had a considerably higher basal metabolism than their controls." Kleiber, Goss and Guilbert ('36) could not detect any systematic influence of phosphorus deficiency on the fasting catabolism of beef heifers, and Goss and Kleiber ('37) noticed that the rate of fasting catabolism for phosphorus-deficient rats was lower than that for the phosphorus-supplied controls. These workers attributed their observation, however, to a general lowering of the nutritive condition of the deficient rats resulting from a diminished food intake. When the food intake of the controls was adjusted to the level of the deficient rats, there was no significant difference in the rate of fasting catabolism per unit of metabolic body size ($kg^{3/4}$).[1] These results were essentially confirmed by Nakamura ('39), who concluded from respiration trials that a deficiency of dietary phosphorus does not affect the basal metabolism of rats.

These observations suggested that the lack of different mineral constituents might affect the energy metabolism in a

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[1] Metabolic body size is defined in this paper as the body weight in kilograms raised to the 3/4 power; $kg^{3/4}$ is the unit of this size. As the unit of surface area we use $kg^{2/3}$.

different way, and it seemed worth while to investigate the metabolic effect of single-ion deficiencies.

The influence of calcium deficiency was selected for the first investigation because two of the authors (see Greenberg, Boelter, and Knopf, '39) were then extensively studying the effects of calcium deprivation.

Greenberg and Boelter [2] observed that extreme lack of calcium in the diet of rats led to a drop in blood-serum calcium, a negative calcium balance, and decreased appetite and rate of growth. It resulted in osteoporosis, in hemorrhages, particularly of the central nervous system which caused paralysis of the hind quarters, and in premature death.

Pedotti ('21) concluded that calcium deficiency lowered the metabolism of rats. His diet (white bread, beef meat after H_2O extraction, and margarine) may, however, have produced an avitaminosis as well as calcium deficiency. Furthermore, he ran his trials at a temperature ranging from 17 to 21°C., whereas the critical temperature for rats, according to Benedict and MacLeod ('29), is 28°C.

Lengyel ('34) lowered the calcium content of rat serum from 10 to 7 mg. % by a vitamin-deficient diet, and raised it again by the addition of cod liver oil. He increased it further by subcutaneous injection of calcium gluconate. He noticed that a low calcium content in the serum was always combined with a raised metabolic rate, and, conversely, a high serum calcium concentration with a low basal metabolic rate.

Goreczky and Ludány ('38), in confirming Lengyel's results, found that human beings reacted to an intravenous injection of a 10% solution of $CaCl_2$ with a sharp drop in the metabolic rate (10 minutes after the injection), followed by a gradual rise, during 3 hours, to the normal level.

The experiments reported here were carried out in order to measure the effect of a dietary deficiency of calcium alone on the rate of fasting catabolism of rats. Upon one group

[2] Thesis for the degree of doctor of philosophy by Muriel D. D. Boelter (1940), University of California Library.

of rats, data were obtained that led to some conclusions regarding the influence of calcium deficiency on the efficiency of food utilization. Similar investigations of magnesium and potassium deficiencies, as affecting metabolism and food utilization, are under way.

EXPERIMENTAL METHODS

Diet. The rats employed in the experiments (Long-Evans strain) were fed the diet shown in table 1.

The calcium-deficient diet contained 10 mg. Ca and 546 mg. P; the control diet, 430 mg. Ca and 437 mg. P per 100 gm. of

TABLE 1

Composition of basal diet and salt mixtures

BASAL DIET		SALT	SALT MIXTURE USED FOR	
			Ca-deficient rats	Control rats
	%		%	%
Casein (acid washed)	22.7			
Fat (Crisco)	22.7	NaCl	0.91	0.66
Sucrose	45.4	KCl	1.15	1.15
Cod liver oil	2.27	Na_2HPO_4	0.91	....
		$Ca_3(PO_4)_2$	....	1.36
Rice bran extract (Vitab. B)	2.73	$MgSO_4$	0.91	0.91
Salt mixture	4.10	Fe citrate	0.12	0.12
			4.00	4.20

Thiamin chloride	5.0 mg.
Synthetic riboflavin	4.0 mg.
Nicotinic acid	50.0 mg.

feed. The first group of six rats was placed on the low-calcium regimen at the age of 20 days; a second group of six rats, at 29 days. For each deficient rat a litter mate control was kept on the same diet with added calcium, the only difference being the composition of the mineral mixture as given in table 1. No restriction was placed on the food intake of the first two groups of deficient and control rats.

This procedure led to differences in body size. The metabolic rate was calculated per unit of the 3/4 power of body weight. This calculation allows the comparison of metabolic rates of

animals which differ in body size (Kleiber, '32). It appeared desirable, however, to check the conclusions drawn from these results by a third group of rats in which the differences in size between pair mates were eliminated by restriction of food intake. The twelve rats of this group were started on the calcium-deficient diet at the age of 30 days. For the first 10 days of this regimen, the food intake was unlimited. The rats were then arranged into six pairs, and one rat of each pair was continued as before on the calcium-deficient diet with unlimited food intake. Its pair-mate received food with the calcium-containing salt mixture. The food intake of the calcium-supplied rat was restricted so that its body weight would remain as nearly as possible that of the deficient pair-mate.

Respiration trials. A series of respiration trials was carried out upon the first group of rats after three of them had been on the deficient diet for a duration of 36 days, and three others for 87 days. The respiration trials were repeated 4 days later. Respiration trials were started upon the second group of rats when six of them had been on the deficient diet for 25 days. The rats were then 54 days old. These trials were repeated twice at weekly intervals.

The first two series of respiration trials on the third group of rats (paired feeding) were carried out during the preliminary period when all twelve rats received calcium-deficient food ad libitum (fig. 1). Four more series of respiration trials were run in the period from 30 to 105 days after the start of the paired feeding. The procedure was the same in all 114 respiration trials reported in this paper. Eighteen hours before the trials, the rats were brought to an air-conditioned room, and maintained at 30°C., where they remained with free access to water but without food. Oxygen consumption and CO_2 production were measured in a multiple apparatus that allows the simultaneous determination of the respiratory exchange of seven individual rats. The apparatus and the procedure of the measurements have been described in detail (Kleiber, '40).

RESULTS

Growth rate and appetite. First among the effects of calcium deficiency is a decrease in, and finally cessation of, growth. The data obtained on our calcium-deficient rats give evidence of this symptom. The mean body weight of five deficient rats [3] of the first group (two weights for each rat taken 4 days apart) was 103 ± 5 gm. The corresponding mean body weight of the six calcium-supplied control rats was 157 ± 8 gm.

The second group of rats behaved similarly. The mean body weight of the six·calcium-deficient rats at the age of 66 days,
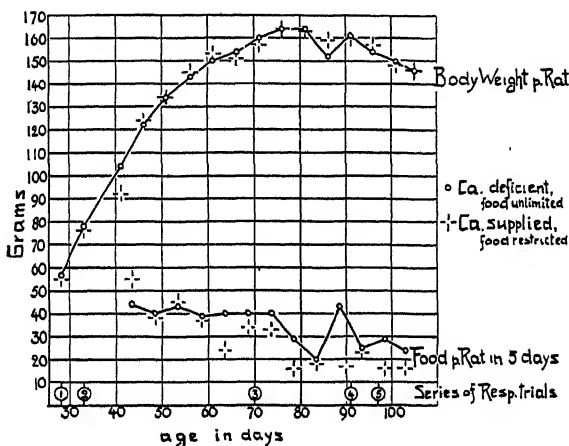
Fig. 1   Body weight and food consumption of Ca deficient and Ca-supplied rats (paired feeding).

after three series of respiration trials, was 123 ± 8 gm., that of the six controls, 163 ± 7 gm.

The decreased rate of growth of the calcium-deficient rats is, to a large extent, the result of decreased appetite. This conclusion is based on the observation that the body weight of the calcium-supplied control rats in the third group could be maintained equal to that of their calcium-deficient pair mates if their food intake was restricted to a level slightly below that of the deficient rats, which were on an unrestricted food

[3] One of the calcium-deficient rats died and its brain was eaten by its cage mates.

intake (fig. 1). The calcium-deficient rats ate 456 gm. of air-dry food in 65 days. The calcium-supplied pair mate received 372 gm. of air-dry food during the same period.

Relative size of parts of body. The deficient rats of the third group were stockier. Their length from nose to end of tail averaged 31.8 ± 0.7 cm., while that of the controls was 35.1 ± 0.6 cm. The deficient rats had, however, thinner bones; their mean femur thickness was 2.6 ± 0.1 mm., while that of the controls was 3.0 ± 0.04 mm. The head, also, was shorter and narrower.

No systematic difference was found in the dry weight of the thyroid and adrenal glands of deficient and control rats. The deficient rats had a higher weight of the liver than the controls: 1.80 ± 0.04 gm. against 1.48 ± 0.09 gm.

Mineral content of rats. As table 2 shows, at the end of the trial, the calcium-deficient rats of the third group (paired feeding) had only half as much ash in their body (2.7 gm. per rat) as did the controls (5.8 gm.). This difference developed even though the calcium-deficient rats ate 9.5 gm. of mineral matter during the entire trial period, and the supplied controls, only 9.1 gm.

The mean calcium content of the dry carcasses of the deficient rats was only about one-third as high as that of the control rats (table 2). The percentage of calcium in the body of the controls (1.01% fresh weight) is practically the same as that found by Sherman and MacLeod ('25) for 90-day old females (0.09%).

The calcium concentration in the blood serum of the calcium-deficient rats was about one-half that of the controls (5.3 mg. per 100 cc. of blood serum for four calcium-deficient rats as against 11.3 mg. per 100 cc. of serum in four calcium-supplied controls). The phosphorus content of the blood was slightly higher in the calcium-deficient rats (9.27 mg. P per 100 cc. serum) than in the controls (8.66 mg. P per 100 cc. serum).

Activity of rats. Among the symptoms of calcium deficiency listed by McCollum, Orent-Keiles and Day ('39) is hyper-irritability. According to Greenberg, Boelter and Knopf

('39), however, lack of calcium alone does not lead to hyper-irritability or tetany. This observation of the last-mentioned authors was confirmed by our work.

In the calcium-deficient rats used in our tests there was nothing to indicate an increased activity in the usual sense of the word. A. H. Smith, assistant of the senior author at Davis, tested the activity by placing all rats together on a table and determining at regular intervals, as most active, those rats that had moved farthest away from the group. He always found that the control rats left the group sooner, and

TABLE 2

Chemical composition of calcium-deficient rats and their pair-fed controls. All data are the means of six rats. Group 3

CATEGORY OF INTEREST	CALCIUM DEFICIENT RATS	CALCIUM SUPPLIED RATS	DIFFERENCE
Body weight:			
Moist, grams	146 ±4.7	146 ±2.4	
Dry, grams	39.7 ±1.6	48.8 ±2.1	9.1 ±2.6
Body analysis:			
Ash in grams	2.7 ±0.04	5.8 ±0.33	3.1 ±0.33
Organic matter, in grams'	37.0 ±1.6	43.0 ±2.2	6.0 ±2.7
Fat in grams	7.1 ±1.7	12.5 ±2.6	5.4 ±3.1
Protein (by difference) in grams	29.9 ±0.9	30.5 ±1.2	0.6 ±1.5
Relative content of calcium in dry carcass in per cent	1.15±0.07	3.02±0.29	1.87±0.30

that the calcium-deficient ones stayed longer where they were placed.

Fasting catabolism. In a first series of respiration trials with the first group of rats, the calcium-deficient rats had a mean daily fasting catabolism of 102.2 ± 27.5 kilocal. per $kg^{3/4}$. The corresponding mean daily rate for the controls was 86.0 ± 5.2 kilocal. per $kg^{3/4}$. The metabolic rate of the calcium-deficient rats was thus considerably and significantly higher than that of the controls, which, as such, was already high compared with the rate of other normal rats. The repetition of these respiration trials on the same rats 4 days later

showed a general decrease in the metabolic rates, but again a greater average daily rate for the calcium-deficient rats (90.2 ± 3.8 kilocal. per $kg^{3/4}$) than for the controls (78.7 ± 3.3 kilocal. per $kg^{3/4}$).

These results were essentially confirmed by three series of respiration trials with the six calcium-deficient and the six control rats of the second group (also on unlimited food intake), carried out when the animals were 54, 60 and 66 days of age. In these trials, the daily metabolic rates of the calcium-deficient rats were 97 ± 2, 100 ± 3, and 92 ± 2, those of the calcium-supplied controls, 88 ± 4, 94 ± 5, and 87 ± 4 kilocalories per $kg^{3/4}$.[4] The difference in the metabolic rates between deficient and control rats is smaller in the second than in the first group.

Possibly the results on the second group of rats are less conclusive than those on the first group because the calcium-deficient rats were erroneously allowed to drink tap water instead of distilled water.[5] Their calcium deficiency, though in evidence, may have been less pronounced than in the first group.

The data obtained in six series of respiration trials on the third group of rats during fasting are summarized in table 3. The first two series were run during the period in which all rats were being fed the same calcium-deficient food ad libitum. The results of these two series were used to select pair mates that were metabolically quasi-equal. On the first trial made after the control and deficient rats were placed on their respective diets, the deficient rats had a higher metabolic rate than their controls (third series). The difference is not statistically significant when expressed per rat (because of the variations within each group), but becomes so when expressed per $kg^{3/4}$.

The metabolic rate of the calcium-deficient rats in the later series remained essentially at the same level when expressed

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[4] See footnote 1.

[5] According to analysis by the chemistry division, the tap water of the University Farm at Davis contains 22 to 31 parts Ca per million, and 44 to 54 parts Mg per million (Bisson and Huberty, '39).

TABLE 3

Fasting catabolism of calcium-deficient and control rats group 3 (paired feeding): results for five pairs of rats

| | SERIES OF RESPIRATION TRIALS | | | | | |
	1 [1]	2 [1]	3	4	5	6
Age in days:	27	33	70	91	97	111
Mean body weight in grams:						
Deficient rat	57 ±2	78 ±4	140 ±3	150 ±9	160 ±5	146 ±3
Control rat	54 ±3	76 ±4	148 ±7	157 ±7	159 ±5	142 ±4
Daily metabolic rate in kg.cal. per rat:						
Deficient rat	12.5±0.6	16.4±0.4	21.5±0.8	20.8±1.4	22.8±0.6	19.7±0.3
Control rat	11.6±0.6	16.4±1.1	19.4±1.2	17.6±0.7	17.2±0.7	14.3±0.2
Per $kg^{3/4}$ in kg.cal.[2]						
Deficient rat	108 ±3	112 ±3	93 ±2	86 ±3	90 ±2	83 ±1
Control rat	101 ±1	113 ±2	81 ±2	71 ±2	68 ±2	62 ±2
Ratio of deficient to control per $kg^{3/4}$:	105 ±5	99 ±1	116 ±3	122 ±3	132 ±4	134 ±2 [3]

[1] In the first two trials, both groups of rats were maintained on the same calcium-deficient diet.

[2] Metabolic body size is defined here as the body weight in kilograms raised to the 3/4 power; $kg^{3/4}$ is the unit of this size. As the unit of body surface $kg^{2/3}$ is used.

[3] Three pairs only.

per rat, and dropped only slightly when expressed per $kg^{3/4}$ (table 3). The metabolic rate of the control rats, however, decreased with increasing age, so that, in the fifth series, when the mean body weight of calcium-deficient and supplied rats was practically the same, the metabolic rate of the calcium-supplied controls was significantly lower than that of their deficient litter mates.

The last column of table 3 shows the average results obtained when the metabolic rate per $kg^{3/4}$ of each calcium-deficient rat was expressed in per cent of the corresponding figure for its litter mate control. As these figures demonstrate, the difference in metabolic rate between calcium-deficient rats and controls increases with age. This increased difference in metabolic rate parallels an increase in the degree of calcium deficiency, which is derived from the curves for weight and appetite (plotted in fig. 1), and from the observation that the deficient rats became less and less active, and occasionally bled from the nose.

We feel justified to draw the conclusion from our observations—on rats fed ad libitum, but particularly on those fed in pairs to maintain equal body weight between deficients and controls—that calcium deficiency increases the rate of fasting catabolism, while decreasing the activity of the rats.

Calcium deficiency and food utilization. The six calcium-deficient rats of the third group gained 42 gm. of body weight per rat in 65 days on 456 gm. of air-dry food. The six calcium-supplied controls gained 54 gm. of body weight per rat in the same time on 372 gm. of air-dry food. The total weight efficiency of the calcium-deficient rats was thus 9.2 gm. gain per 100 gm. of air-dry food consumed; for the calcium-supplied rats, 14.5 gm. gain per 100 gm. air-dry food.

The heat of combustion of the rat feed was calculated from the chemical composition, using 5.86 kilocal. per gram casein, 9.4 kilocal. per gram fat, and 4.0 kilocal. per gram sucrose. By this calculation, 100 gm. rat feed with 92 gm. dry matter had a heat of combustion of 5.15 kilocal. During the 65 days of the trial, the calcium-deficient rats consumed 84 gm. more

air-dry food per rat than the controls. This difference in food intake amounts to 433 kilocal. of food energy. The calcium-supplied controls, moreover, gained 5.4 gm. more body fat and 0.6 gm. more body protein per rat than their calcium-deficient pair mates. The energy of this extra gain amounts to 55 kilocal. per rat. Thus, the deficient rats wasted 433 + 55 = 488 kilocal. more energy per rat in 65 days, or 7.5 kilocal. more per day than the controls. This amounts to 25.5% of the total food energy consumed by the calcium-supplied rats (372 × 5.15 = 1916 kilocal.) during the experimental period.

This extra waste by the deficient rats may be explained partially by their higher fasting catabolism. The mean difference for three series of respiration trials (series 3, 4 and 5, table 3) was 3.6 kilocal. of heat lost per rat per day. Since one may reasonably assume that this difference in the fasting catabolism between deficient and supplied rats was smaller at the earlier stages of the deficiency, one may deduce that no more than one-half of the extra waste of food energy observed in the calcium-deficient rats is accounted for by the increase in the basal metabolism. Therefore, it may be concluded that calcium deficiency not only decreased the total efficiency of food utilization by increasing the basal metabolic rate, but also decreased the partial efficiency of food utilization.[6] This may mean that calcium-deficiency leads to a lower digestibility or to a greater excretion of incompletely oxidized products in the urine. It may also mean a higher calorigenic action or, of course, a combination of these three effects.

Kriss and Smith ('38) did not find a significant difference in the calorigenic effect of a low salt diet compared with a normal salt diet fed to rats. They conclude from their results that "the increases in the basal metabolism caused by the mineral deficiency can fully account for the relatively greater increases in the total heat production of the low-salt rats in the feeding periods."

[6] Total efficiency of energy utilization is defined as the quotient
$\frac{\text{Total gain of body energy}}{\text{Total energy of food consumed}}$, the partial efficiency as the quotient
$\frac{\text{Change in gain of body energy}}{\text{Corresponding change in energy of food consumed}}$

One is, however, hardly justified in applying the results with a low salt diet directly to our investigation of the effect of a deficiency of calcium alone.

The protein content of the rat feed was calculated from the results of a Kjeldahl determination by multiplying the nitrogen content by 6.38 which is the factor given for casein in the Official Methods for Agricultural Chemists ('35). By this calculation, 100 gm. of air-dry food contained 19.1 gm. protein. The calcium-deficient rats, having eaten 84 gm. more of air-dry food during 65 days than did the calcium-supplied controls, consumed 16.0 gm. more protein per rat. Since, moreover, the deficient rats gained 0.6 gm. less body protein per rat, they wasted 16.6 gm. more protein per rat than the controls. This extra waste amounts to 0.25 gm. protein per rat per day.

The extra waste of food energy, resulting from calcium deficiency, amounted to 25% of the energy intake, the extra waste of protein to 23% of the protein intake, of the calcium-supplied controls. This comparison suggests that calcium deficiency affected the utilization of food protein to practically the same degree that it affected the utilization of food energy.

SUMMARY

1. Rats were fed a diet containing only 10 mg. calcium per 100 gm. of food. After 2 months, their mean body weight was approximately 30% below the body weight of litter mate controls fed ad libitum on the same diet, but with added calcium. A considerable decrease in appetite on the calcium-deficient diet is mainly responsible for the retarded growth.

2. The body weight of calcium-supplied control rats could be kept equal to that of their calcium-deficient pair mates by restricting their food intake through paired feeding to a level slightly, but significantly, below that of the calcium-deficient rats.

3. The calcium-deficient rats were shorter, with a smaller skull, and a finer skeleton than their pair-fed controls. The

liver dry weight of the deficient rats was significantly higher than that of the controls. The carcasses of the deficient rats contained only one-half as much ash, and only one-third as much calcium, as that of the calcium-supplied controls. The calcium concentration of the blood serum of the deficient rats was reduced to one-half that of the controls. The calcium-deficient rats were less active than the controls.

4. The rate of fasting catabolism per unit of 3/4 power of body weight of the calcium-deficient rats, kept in an environment of 30°C. for 18 hours before, as well as during, the respiration trials, was consistently higher for the calcium-deficient rats than for their calcium-supplied controls. This result was obtained with two groups of rats all of which were fed ad libitum, in which case the calcium-deficient rats had a considerably smaller body weight than their calcium-supplied litter mate controls. The increase of metabolic rate resulting from calcium deficiency was confirmed by a third group of rats in which the pair mates were kept equal in body weight by restricting the food intake of the calcium-supplied controls. In the pair-fed group, the difference in the metabolic rate between calcium-deficient and calcium-supplied rats seemed to increase as the deficiency became more severe. The metabolic rate of the deficient rats in three successive series of trials was 116 ± 3, 122 ± 3, and $132 \pm 4\%$ of the corresponding rate of the controls.

5. The total efficiency of utilization of food energy [7] was decreased by calcium deficiency, not only by a lowering of the food intake and by a higher basal metabolism, but also by a lower partial efficiency. This may mean a greater loss of unoxidized material in feces and urine, or a higher calorigenic action.

[7] See footnote 6.

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ADAPTATION OF THE GROWING RAT TO THE INGESTION OF A CONSTANT CONCENTRATION OF FLUORINE IN THE DIET [1,2]

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THREE FIGURES

It has been repeatedly shown that the animal body when faced with adverse nutritive conditions adapts itself to them in such a way as to minimize their deleterious effects. When its food supply is restricted to borderline or inadequate levels of one or more nutrients, it uses its food with greater economy, either by lowering its nutritive requirements or by using more efficiently what nutritive material it secures. Young rats on a restricted intake of protein require less and less of both protein and energy for the maintenance of body weight (Jackson, '37). In a similar manner, human subjects on an inadequate intake of energy adapt themselves to the restricted diet by a lowering of the basal metabolism and possibly in other ways, with no observed detriment to health (Benedict et al., '19). The dairy cow, even while producing milk, adapts herself to a low level of calcium intake with no impairment of health, breeding efficiency or milk and butterfat production over a period of 5 or 6 years (Fitch et al., '32;

Palmer et al., '35). "Balance trials furnished the explanation of these results in indicating an ability of dairy type cows to adjust themselves to the Ca content of the ration and to conserve the quantity ingested when this was limited." In a similar manner, growing children habituated to a low-calcium diet have been found to store up to 89% of an intake (0.2 to 0.25 gm.) only one-fifth to one-fourth of that recommended in current dietary standards (Nicholls and Nimala-suriya, '39). At least a partial explanation of this adaptation to an intake of calcium otherwise incompatible with maximum retention may be found in the fact that the efficiency of calcium utilization is dependent upon the degree of calcium saturation of the tissues, the efficiency being greater with depleted than with replenished stores (Rottensten, '38). The adaptive powers of the body thus may alleviate, or within limits, entirely obviate the penalties of undernutrition.

In the case of harmful dietary constituents also, the adaptive powers of the body come into play. As examples only, the acquisition of a tolerance to organic arsenicals (Kuhs, Longley and Tatum, '39) and to alcohol (Lévy, '35) may be cited. Presumably this tolerance involves either an increased efficiency in the elimination from the body by excretion or oxidation of the deleterious constituent, or the development of a hyposensitivity to it, or both.

There is little information available concerning the adaptation of the body to dietary fluorine. Among the few studies on record in which the balance of fluorine was determined with experimental animals is the investigation of Brandl and Tappeiner (1891) on a dog. The animal was fed increasing amounts of sodium fluoride from 0.1 to 0.9 gm. daily and the output of fluorine in urine and feces was determined in successive 3-week periods. In the first 3 weeks no fluorine was detected in the excreta, which thereafter contained rapidly increasing amounts. Making allowance for the crude analytical method for fluorine used, the findings nevertheless indicate a rapidly increasing efficiency of the body in eliminating ingested fluorine. In an investigation of the relative toxicity

of fluorine administered in water and in food, the writers have recently shown (Lawrenz, Mitchell and Ruth, '39 b) that in balance periods 8 weeks apart a considerably greater proportion of the ingested fluorine was excreted in the second than in the first period, the averages for four rats being 71 and 49%, respectively. These few data also point to an increasing efficiency in the elimination of fluorine with increasing duration of fluorine feeding.

The purpose of the investigation to be reported in this paper was to secure additional and more complete information of the adaptation of the growing rat to diets containing constant concentrations of fluorine. In particular, it was desired to follow closely the efficiency of the excretion of fluorine in successive short periods of time, and to determine whether the degree of adaptation, if such occurred, would depend upon concentration of fluorine in the diet. Since this investigation is one of a series relating to the fluorine hazard incident to the consumption of sprayed fruit, the source of the fluorine was the spray chemical, synthetic cryolite, and the concentrations of dietary fluorine studied were low, not exceeding 15 p.p.m.

EXPERIMENTAL METHODS

The balance of fluorine on three experimental diets was compared in successive weekly or biweekly periods. The basal diet consisted of ground wheat 62%, dried whole milk 33%, fortified cod liver oil 1.5%, wheat germ oil 0.5%, sodium chloride 1%, and 2% of a modification of the Wesson ('32) salt mixture, consisting of the withdrawal of sodium fluoride and the addition of small amounts of cobalt chloride and zinc carbonate. The diets to be compared were prepared by adding to the basal diet synthetic cryolite in amounts to provide 3, 6 and 12 p.p.m. of fluorine in addition to that present in the basic ingredients. The first diet always contained over 4 p.p.m. of fluorine, and the second and third diets about 6.5 and 12.5 p.p.m., respectively. Apparently there was a greater loss of the cryolite dust during mixing of the latter two rations than of the former.

In comparing these diets, six trios of rats were selected as experimental animals. The rats in each trio were taken from the same litter and were of the same sex and approximately of the same body weight. Five of the trios were young rats weighing initially between 40 and 50 gm. The sixth trio was started on experiment 4 weeks later, because of a lack of metabolism cages, and weighed initially about 137 gm. During this 4-week interim the three rats were kept on a diet to which no fluorine was added, and fed in amounts to maintain approximate equality in weight. Throughout the experimental feeding period, the rats in each trio were fed the same amount of food.

The first four collection periods were of 6 or 7 days duration each. All subsequent periods were 14 days in length.

The rats were kept in metabolism cages identical with those previously described (Lawrenz, Mitchell and Ruth, '39 b). The feces of the various experimental periods were separated by the use of Fe_2O_3 as a marker. The collection of feces and urine and their analysis for fluorine have been described in detail in the paper just cited. As there stated, the collections of urine were filtered through glass wool to remove contaminations of hair, bits of food and other debris. This accumulated waste from each rat was saved and analyzed for fluorine at the end of the experiment.

The rats were continued on experiment from 22 to 32 weeks. Most of the rats developed "sniffles" after 6 months, and the experiment on any trio of rats was terminated if the respiratory disease became serious enough to impair appetite.

At the termination of the feeding period, the rats were killed, the body length and empty weight determined, and the fluorine content of skeleton, teeth and soft tissues determined as previously described (Lawrenz, Mitchell and Ruth, '39 a).

DISCUSSION OF EXPERIMENTAL RESULTS

During the course of the feeding experiment no ill effects of the fluorine supplements were noted. There was no tendency

TABLE 1

Growth data and fluorine analyses of tissues

Trio number and sex	1 ♂			2 ♀			3 ♀		
Rat number	181	182	183	184	185	186	187	188	189
Fluorine added to ration, p.p.m.	3	6	12	3	6	12	3	6	12
Feeding period in days	155	155	155	225	225	225	225	225	225
Total food consumed, gm.	1767	1767	1767	2070	2070	2070	1918	1918	1918
Total gain in body weight, gm.	301	303	314	216	206	207	200	210	214
Final empty body weight, gm.	345	346	358	258	240	249	242	254	259
Total weight of bones,[1] gm.	15.302	15.972	15.663	14.278	15.064	14.427	12.575	12.036	16.278
Total weight of teeth,[2] gm.	0.484	0.495	0.491	0.494	0.525	0.454	0.510	0.523	0.563
Fluorine in bones, p.p.m.	170	278	510	205	296	565	218	295	510
Fluorine in teeth, p.p.m.	41	81	187	49	86	207	55	89	175
Total fluorine ingested, mg.	7.796	11.346	22.011	8.975	13.214	25.873	8.305	12.196	23.835
Fluorine in bones, mg.	2.601	4.440	7.988	2.927	4.459	8.152	2.741	3.816	8.302
Fluorine in teeth, mg.	0.020	0.040	0.092	0.024	0.045	0.094	0.028	0.047	0.098
Fluorine in soft tissues, mg.	0.101	0.153	0.226	0.195	0.251	0.405	0.116	0.187	0.299
Total fluorine in carcass, mg.	2.722	4.633	8.306	3.146	4.755	8.651	2.885	4.050	8.699
Estimated fluorine retention,[3] mg.	2.493	4.409	8.077	2.928	4.531	8.433	2.667	3.821	8.465
Per cent retention of fluorine	32.0	38.8	36.7	32.6	34.3	32.6	32.1	31.3	35.5
Per cent of retained fluorine in bones	95.5	95.8	96.2	93.0	93.8	94.2	95.0	94.2	95.4
Per cent of retained fluorine in teeth	0.74	0.86	1.11	0.76	0.95	1.09	0.97	1.16	1.13

	4 ♂			5 ♂			6 ♀			AVERAGES		
Trio number and sex	190	191	192	193	194	195	196	197	198			
Rat number												
Fluorine added to ration, p.p.m.	3	6	12	3	6	12	3	6	12	3	6	12
Feeding period in days	197	197	197	168	168	168	198	198	198	195	195	198
Total food consumed, gm.	2063	2063	2063	1680	1680	1680	1751	1751	1751	1875	1875	1875
Total gain in body weight, gm.	276	247	305	286	265	252	86	103	91	227	222	230
Final empty body weight, gm.	324	295	353	328	307	295	225	240	226	287	282	290
Total weight of bones,[1] gm.	15.256	15.344	15.563	14.837	13.835	14.239	14.152	14.717	15.092	14.400	14.645	15.210
Total weight of teeth,[2] gm.	0.543	0.615	0.575	0.517	0.504	0.477	0.522	0.533	0.485	0.512	0.533	0.507
Fluorine in bones, p.p.m.	170	265	575	176	270	581	182	250	477	187	276	536
Fluorine in teeth, p.p.m.	34	73	166	51	103	192	53	100	197	47	89	187
Total fluorine ingested, mg.	9.010	13.209	25.797	7.405	10.805	21.031	7.671	11.257	21.732	8.192	12.004	23.379
Fluorine in bones, mg.	2.594	4.066	8.949	2.611	3.735	8.273	2.576	3.679	7.199	2.675	4.033	8.144
Fluorine in teeth, mg.	0.019	0.045	0.096	0.027	0.052	0.092	0.028	0.053	0.096	0.024	0.047	0.095
Fluorine in soft tissues, mg.	0.112	0.141	0.466	0.136	0.200	0.220	0.155	0.133	0.182	0.136	0.178	0.300
Total fluorine in carcass, mg.	2.725	4.252	9.511	2.774	3.987	8.585	2.759	3.865	7.477	2.835	4.258	8.539
Estimated fluorine retention,[3] mg.	2.476	4.003	9.262	2.556	3.769	8.361	2.548	3.654	7.266	2.612	4.031	8.310
Per cent retention of fluorine	27.5	30.3	35.9	34.5	34.9	39.7	33.2	32.4	33.4	32.0	33.7	35.6
Per cent of retained fluorine in bones	95.2	95.6	94.1	94.1	93.7	96.4	93.4	95.2	96.3	94.4	94.7	95.4
Per cent of retained fluorine in teeth	0.70	1.06	1.01	0.97	1.30	1.07	1.01	1.37	1.28	0.86	1.12	1.12

[1] Dry fat-free weight.

[2] Weight after drying at 110°C.

[3] The initial fluorine content of the rats was estimated at 5.2 p.p.m. except for trio 6, for which the value of 2.1 p.p.m. was used.

where p is the percentage of the fluorine intake excreted at time t measured from the initiation of the diet, A is the maximum percentage toward which the curve is moving, B is the total gain in p made in reaching the maximum value A, and k is a constant expressing the fractional decline in the rate of increase of p. This equation has been fitted to the three sets of data by a method suggested by Lipka ('18, p. 142),
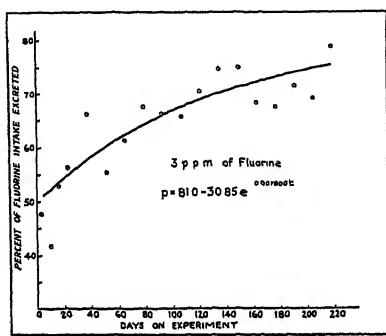
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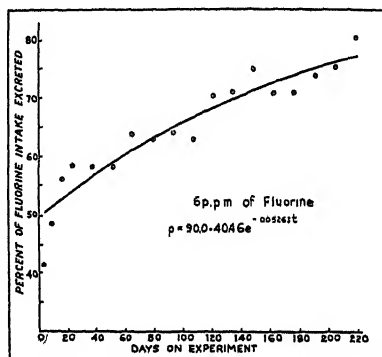
2

Fig. 1   The relationship between the percentage excretion of fluorine in urine and feces, and the elapsed time since fluorine feeding was initiated, in the case of rats receiving the diet containing 3 p.p.m. of added fluorine.

Fig. 2   The relationship between the percentage excretion of fluorine in urine and feces, and the elapsed time since fluorine feeding was initiated, in the case of rats receiving the diet containing 6 p.p.m. of added fluorine.
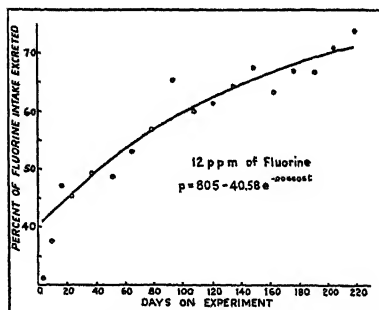
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Fig. 3   The relationship between the percentage excretion of fluorine in urine and feces, and the elapsed time since fluorine feeding was initiated, in the case of rats receiving the diet containing 12 p.p.m. of added fluorine.

which is simple but quite satisfactory for such small sets of variable data. It is evident from the charts that the curves thus obtained trace the course of the relationship between percentage fluorine excretion and time, and therefore that this relationship is one of diminishing increment. This means that the percentage fluorine excretion increases toward a maximum value but at a constantly diminishing rate. This maximum percentage varies from 80.5 to 90 for the three groups of data.

TABLE 2

Average percentage excretion of fluorine in successive periods

LENGTH OF PERIODS IN DAYS	RAT 181	RAT 184	RAT 187	RAT 190	RAT 193	RAT 196
3 p.p.m. of fluorine added to diet:						
27	54.7	51.1	39.7	55.5	50.4	57.7
56	63.6	58.5	63.8	61.8	57.1	70.8
70	70.6	71.5	59.9	68.9	69.0	72.5
70	...	66.4	72.3	72.1 [1]	...	...
	RAT 182	RAT 185	RAT 188	RAT 191	RAT 194	RAT 197
6 p.p.m. of fluorine added to diet:						
27	48.7	48.9	54.9	47.3	50.9	56.4
56	61.9	57.2	58.4	60.4	59.2	67.8
70	67.4	62.8	71.7	71.3	65.4	73.2
70	...	71.0	75.4	72.5 [1]	...	...
	RAT 183	RAT 186	RAT 189	RAT 192	RAT 195	RAT 198
12 p.p.m. of fluorine added to diet:						
27	41.4	32.6	37.8	39.3	45.9	45.7
56	51.1	51.9	51.8	51.0	44.5	60.6
70	62.6	62.8	63.2	61.6	60.5	70.3
70	...	67.2	69.5	64.1 [1]	...	...

[1] This period lasted only 56 days.

That this increase is highly significant is proved by the fact that it is evident in the metabolism data for each of the eighteen rats in the experiment. The disturbing effect of the large period-to-period variation in the percentage of the fluorine intake excreted is minimized by averaging together a number of individual period results and thus dividing the experiment into three or four subperiods. This has been done in the construction of table 2. It may be seen from a study

of this table that for each rat there is a progressive increase in the percentage with only two exceptions, i.e., rats 184 and 195, which exhibited decreases from the third to the fourth period, in the former case, and from the first to the second period in the latter.

The increase with elapsed time in the percentage of the fluorine intake eliminated from the body was evident in both fecal and urinary excretions but to a somewhat greater degree in the fecal excretion. On the average, about 46% of the excreted fluorine appeared in the urine and 54% in the feces.

The diminishing rate of increase in the percentage excretion may be considered the result of a physiological adaptation of the organism to a foreign and potentially harmful dietary ingredient. The excretion-time relationship is similar to other adaptation phenomena, such as the dark adaptation of the human eye as described by the threshold intensities of light (Wald, Jeghers and Arminio, '38), and the compensatory hypertrophy of the kidney after unilateral nephrectomy (Smith and Moise, '27) and superimposed graded increases in protein intake (MacKay, Addis and MacKay, '38, fig. 1). Advancing age of the rats may have modified the picture to some extent, since the rats in trio 6 (nos. 196, 197 and 198), put on experiment at a much greater initial weight, each excreted in general a greater percentage of the ingested fluorine than the other rats on the same rations (table 2). On the other hand, these half-grown rats exhibited essentially the same excretion-time relationship as the other rats started on experiment at only one-third of their initial weight.

The curves in figures 1, 2 and 3 indicate certain differences among the three groups of rats on the three experimental rations with reference to the percentage of ingested fluorine excreted. In general the observed percentage decreased as the fluorine supplement to the ration was raised from 6 to 12 p.p.m., while no consistent effect was produced on raising the supplemented fluorine from 3 to 6 p.p.m. Out of ninety-four possible comparisons between individual rats and individual experimental periods, fifty comparisons of the 3 and 6 p.p.m.

levels showed a greater percentage excretion of fluorine for the lower fluorine level; in the comparison of the 3 and 12 p.p.m. levels, seventy-three cases favored the lower fluorine level, while in the comparison of the 6 and 12 p.p.m. levels, seventy-seven cases favored the lower fluorine level. The most probable outcome in all comparisons if fortuitous factors alone operated would be forty-seven cases favoring the lower fluorine level and as many favoring the higher. The deviations from the ideal may be compared with the standard deviation of the binomial distribution of ninety-four events that may happen with equal probability in either of two ways, i.e., $\sqrt{0.5 \times 0.5 \times 94} = 4.85$. The deviation of 50 from 47 is considerably less than the standard deviation and hence may well have resulted from fortuitous factors only. On the other hand, deviations of $73 - 47 = 21$ and of $77 - 47 = 30$ are four to six times the standard deviation and hence may reasonably be considered the results of differences in imposed experimental conditions. From this analysis, it may be concluded that the percentage excretion of fluorine on the highest fluorine level tested is significantly less than that on the two lower levels, while between the latter two no significant difference exists.

The fluorine balance data may be compared with the results of the carcass analyses (table 1) with reference to the percentage of retained fluorine. In making this comparison, the total of excreted fluorine for each rat was increased by the amount of fluorine found in the debris filtered from its urine collections, averaging 40, 55 and 58 mcg. for the rats on the three experimental rations. These values deducted from total fluorine intake would give an indirect determination of the fluorine retained, which is then expressed as a percentage of the intake. With the second set of data, the retained fluorine would equal the difference between the fluorine recovered in the carcasses and the initial contents of fluorine estimated from the initial body weights of the rats and the concentration of fluorine found in the check rat litter mates sacrificed at the beginning of the experiment. For the five trios started

at 40 to 50 gm. in weight, the corresponding check rats analyzed from 4.7 to 5.7 p.p.m. of fluorine with an average of 5.2. For trio 6 started at about 140 gm., the check rat analyzed 2.1 p.p.m. of fluorine. A comparison of the results of these two methods of estimating the percentage of retained fluorine is made in table 3.

The estimates of fluorine retention expressed as per cents of the intakes obtained from carcass analysis were smaller for all rats but two than the estimates based on the balance data. The average differences for the rats on the three levels of fluorine were 2.6, 1.1, and 7.1, respectively, equivalent to percentage deviations of 8.1, 3.3 and 19.9 from the average values obtained from the carcass analyses. Quite probably the balance data are in error on account of the much greater number of possibilities for loss of fluorine during the many manipulations involved. For the two lower levels of fluorine intake the errors are seemingly not excessive, but for the highest level the error is considerable. Subsequent tests showed that the microtechnic employed in analyzing the urine and feces of the rats receiving 12 p.p.m. of fluorine in their food was demonstrably in error because of the relatively large amounts of fluorine involved. Probably for this reason the results are about 5% too low.

A statistical analysis by the method of Student ('08) of the percentage retentions of fluorine estimated from the amounts of fluorine recovered from the carcasses (table 3) indicates that while the average percentage of retained fluorine increased with increasing fluorine intake (31.98, 33.68 and 35.65, respectively) only the average difference between the lowest and the highest levels (M = 3.66, s = 2.94, P = 0.020) is significant, the value of P indicating the probability of a fortuitous outcome. Possibly the number of animals used in this experiment is not great enough to establish the significance of the intermediate differences. We may conclude, however, that the effect of an increasing concentration of fluorine in the diet for the range of concentrations studied is to increase the percentage of fluorine retained. This would

TABLE 3

A comparison of data of fluorine balance and of carcass analysis

Trio number and sex	1 ♂			2 ♀			3 ♀		
Rat number	181	182	183	184	185	186	187	188	189
Fluorine added to ration, p.p.m.	3	6	12	3	6	12	3	6	12
Fluorine retained during experiment, estimated from									
(a) Balance data, mg.	2.562	4.092	9.777	3.182	4.911	10.794	2.890	3.854	9.493
(b) Carcass analysis, mg.	2.493	4.409	8.077	2.928	4.531	8.433	2.667	3.821	8.465
Per cent of fluorine intake retained, estimated from									
(a) Balance data	32.9	36.1	44.4	35.4	37.2	41.7	34.8	31.6	39.8
(b) Carcass analysis	32.0	38.8	36.7	32.6	34.3	32.6	32.1	31.3	35.5

Trio number and sex	4 ♂			5 ♂			6 ♀			AVERAGES		
Rat number	190	191	192	193	194	195	196	197	198			
Fluorine added to ration, p.p.m.	3	6	12	3	6	12	3	6	12	3	6	12
Fluorine retained during experiment, estimated from												
(a) Balance data, mg.	3.159	4.441	11.134	2.761	4.058	9.882	2.457	3.680	8.652	2.835	4.173	9.971
(b) Carcass analysis, mg.	2.476	4.003	9.262	2.556	3.769	8.361	2.548	3.654	7.266	2.612	4.031	8.310
Per cent of fluorine intake retained, estimated from												
(a) Balance data	35.1	33.6	43.2	37.3	37.6	47.0	32.0	32.7	39.8	34.6	34.8	42.7
(b) Carcass analysis	27.5	30.3	35.9	34.5	34.9	39.7	33.2	32.4	33.4	32.0	33.7	35.6

indicate a less efficient adaptation to fluorine ingestion for the higher levels of intake.

From the data of table 1, it appears that the imposed increases in the concentration of dietary fluorine brought about increases in the concentration of fluorine in bones and teeth. The data for the soft tissues are not considered entirely reliable, because of more or less obvious contamination with small particles of bone and cartilage. The teeth are more readily affected than the bones, since at the lowest level of fluorine intake the concentration of fluorine in the bones averaged four times that in the teeth, while at the highest level it averaged only 2.9 times. Only the teeth produced on the highest level of fluorine exhibited the characteristic striations under low-power magnification.

CONCLUSIONS

Growing rats adapt themselves to the continuous ingestion of low levels of fluorine by excreting greater and greater proportions of the ingested fluorine in feces and urine. The rate of adaptation decreases with elapsed time in accordance with the equation describing the curve of diminishing increments. Maximum adaptation corresponds to increases of 60 to 100% in the proportion of ingested fluorine that is eliminated by the intestine and the kidney.

This adaptation involves the excretory capacity of both kidney and intestine, that of the latter to a somewhat greater extent.

Within the range of dietary concentration of fluorine studied (4 to 12.5 p.p.m.), the adaptation of the growing rat to this potentially deleterious dietary ingredient is somewhat less efficient the greater the proportion of fluorine in the consumed food.

Increasing consumption of fluorine occasions greater increases in the concentrations of this element in the teeth than in the bones.

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THE PREVENTION OF NUTRITIONAL MUSCULAR DYSTROPHY IN GUINEA PIGS WITH VITAMIN E [1]

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Goettsch and Pappenheimer ('31) in studying the nutritional role of vitamin E in herbivora observed an early appearance of extensive skeletal muscle degeneration in guinea pigs and rabbits reared on natural food diets in which the vitamin E had been destroyed by $FeCl_3$. Although their diets were deficient in vitamin E, the administration of wheat germ oil did not appear to prevent the development of the deficiency disease.

Madsen, McCay and Maynard ('33, '35), in feeding synthetic rations containing cod liver oil to guinea pigs and rabbits, observed a muscular dystrophy similar to if not identical with that described by Goettsch and Pappenheimer. The substitution of a cod liver oil concentrate or of carotene and irradiated yeast for cod liver oil delayed but did not prevent the muscle degeneration. Madsen ('36) found that the inclusion of cotton seed oil in the diet resulted in a higher degree of protection against the dystrophy. McCay, Paul and Maynard ('37) reported that the muscle lesions were not produced when hydrogenated cod liver oil replaced the natural product.

Morgulis and Spencer ('36), Morgulis, Wilder and Eppstein ('38) and Morgulis ('38) were able to show that the Goettsch and Pappenheimer diet was deficient in two factors, one water-

soluble (present in wheat germ, alfalfa, lettuce and yeast) and the other fat-soluble (supplied by wheat germ oil, and in all probability vitamin E). Mattill ('38) has found that the dystrophy can be prevented by the inclusion of 2% wheat germ oil in a synthetic diet containing no source of vitamin E. He has suggested that autoxidative rancidity of the animal fats included in the dystrophy-producing diets may be the principal cause of the disorder, and that the vitamin E in the diets would serve as an anti-oxidant (Olcott and Emerson, '37) as well as a vitamin. Cummings and Mattill ('31) had previously shown that oxidative reactions initiated by the autoxidation of cod liver oil are destructive to vitamin E. Barrie ('38) and Goettsch and Ritzmann ('39) found that alpha tocopherol prevented the development of paralysis in the suckling young of vitamin E-low mothers, shown by Olcott ('38) to be due to a degeneration of the cross-striated musculature.

Mackenzie and McCollum ('39) have demonstrated the curative effect of alpha tocopherol with rabbits maintained on a modified Goettsch and Pappenheimer diet containing 10% ether-extracted wheat germ. Shimotori, Emerson and Evans ('39) have reported the protective effect of alpha tocopherol with guinea pigs reared on the Madsen, McCay and Maynard diet, modified in that it contained 10% yeast; and Morris ('39) has recently shown the curative effects of alpha tocopherol with rabbits reared on natural-food diets supplemented with cod liver oil.

Mattill ('39) has explained the production of dystrophy in herbivora as follows: "Herbivorous animals have a large cecum where the food remains long enough for autoxidative changes to progress farther and more rapidly than in omnivorous animals such as rats. From this point of view the long search for a toxic factor in cod liver oil, and for cures of the disorders produced thereby (cattle, rabbits, poultry) may have been following a wrong trail."

The results herein reported deal with the prevention of nutritional muscular dystrophy in guinea pigs by the prophylactic administration of vitamin E.

EXPERIMENTAL

The diets used in these studies are shown in table 1. The diets were prepared by adding water to the agar-agar to make a gel. The remainder of the ingredients were then added and the mixture was run through a meat grinder. The cellulose was added after grinding and the mixture was allowed to dry. All diets were supplemented with 5 cc. of orange juice daily.

A preliminary study was undertaken to ascertain whether whole wheat germ would prevent the dystrophy in guinea pigs subsisting on cod liver oil-containing diets. The cod liver oil

TABLE 1

	DIET I	DIET II	DIET III	. DIET IV
	%	%	%	%
Casein (commercial)	15	15	15	15
Regenerated cellulose (Sylphrap)	15	15	15	15
Cornstarch	35	30	40	35
Sucrose	14	14	14	14
Agar-agar	5	5	5	5
Wheat germ	5	10	..	..
Yeast [1]	5	5	5	10
Lard	2	2	2	2
Cod liver oil [2]	2	2	Fed separately	Fed separately
Salt mixture [3]	2	2	2	2

[1] Yeast foam powder, from the Northwestern Yeast Company, Chicago, Ill.
[2] From E. R. Squibb & Sons, New York.
[3] Hubbell, Mendel and Wakeman ('37).

was incorporated into the diet to the extent of 2%, a quantity reported by Madsen et al. as sufficient to cause a severe dystrophy within a period of 2 months. Whole wheat germ was incorporated in the diet at two levels, namely, 5 and 10% (diets I and II). The guinea pigs were gradually accustomed to the ration by allowing a preliminary period of a few weeks to elapse during which the diet was gradually changed from the stock to the synthetic ration.

The animals maintained on these diets supplemented with 5 cc. of orange juice daily demonstrated that, in the presence of cod liver oil, 5 and 10% of wheat germ prevented dystrophy. As indicated in table 2, all of the animals appeared normal

when sacrificed at the periods indicated. However, the muscle creatine was low in three of the four guinea pigs receiving 5% wheat germ (method of Rose, Helmer and Chanutin, '27). Goettsch and Brown ('32), Morgulis and Spencer ('36) and Fenn and Goettsch ('37) have found muscle creatine values to be invariably low in dystrophic rabbits, a finding that has been likewise observed in both suckling and adult rats with muscular dystrophy resulting from E avitaminosis.

TABLE 2
The anti-dystrophic activity of wheat germ

DIET	DESIGNA-TION OF GUINEA PIG	DAYS ON EXPERI-MENT	MAXIMUM WEIGHT	APPARENT CONDITION WHEN SACRIFICED	MUSCLE CREATINE PER 100 GM. FRESH MUSCLE
			gm.		*mg.*
I (5% wheat germ)	1	278	676	Normal	267
	2	231	652	Normal	343
	3	231	504	Normal	277
	4	259	906	Normal	298
II (10% wheat germ)	5	280	650	Normal	407
	6	208	706	Normal	372
	7	350	740	Normal	388
	8	230	536	Normal	360
Stock pellets + carrots	6 guinea pigs				362–438

The next experiment was planned to test the possible protective action of wheat germ oil in the prevention of muscular dystrophy. Diet III, a slight modification of the ration employed by Madsen, McCay and Maynard ('35), was used in this experiment.

The guinea pigs were divided into the following groups based upon the supplements received daily (in addition to the orange juice): (1) 0.5 cc. cod liver oil [2]; (2) 0.5 cc. cod liver oil and 0.5 cc. wheat germ oil;[3] and (3) 0.5 cc. cod liver oil and 0.75 cc. wheat germ oil.

[2] The cod liver oil was considered to represent the remaining 2% of the diet.

[3] The oil when assayed for vitamin E in a single dose of 0.5 gm. with young female rats of proved sterility enabled four out of four test animals to bear normal sized litters of young of normal weight.

The results of these experiments are tabulated in table 3. The ten control guinea pigs (group 1) developed muscular dystrophy and were almost moribund when killed. The first detectable symptom was a general loss of muscle tone. Difficulty was encountered in moving the hind limbs. The guinea pigs exhibited a marked delay in righting when placed on their backs. There was a striking decline in weight, and in the terminal stages the animals, being unable to stand, would lie on

TABLE 3

The anti-dystrophic activity of wheat germ oil and of alpha tocopherol

DIET AND SUPPLEMENT	DESIGNA-TION OF GUINEA PIG	DAYS ON EXPERI-MENT	MAXIMUM WEIGHT	APPARENT CONDITION WHEN SACRIFICED	MUSCLE CREATINE PER 100 GM. FRESH MUSCLE
			gm.		*mg.*
III + 0.5 cc. cod liver oil	9	68	392	Paralyzed	252
	10	80	498	Slightly paralyzed	270
	11	86	440	Paralyzed	117
	12	71	314	Paralyzed	154
	13	104	580	Paralyzed	165
	14	30	...	Paralyzed	288
	15	100	480	Paralyzed	195
	16	38	404	Slightly paralyzed	257
	17	61	420	Paralyzed	134
	18	50	474	Paralyzed	255
III + 0.5 cc. cod liver oil and 0.5 cc. wheat germ oil	19	355	690	Normal	396
	20	355	550	Normal	400
	21	256	616	Normal	428
III + 0.5 cc. cod liver oil and 0.75 cc. wheat germ oil	22	301	552	Normal	408
	23	301	608	Normal	390
	24	301	500	Normal	413
IV + 0.5 cc. cod liver oil	25	26	206	Paralyzed	...
	26	54	291	Paralyzed	...
	27	37	358	Paralyzed	...
	28	35	272	Paralyzed	...
	29	35	322	Paralyzed	...
	30	20	302	Paralyzed	...
	31	67	333	Paralyzed	...
	32	20	...	Paralyzed	...
IV + 0.5 cc. cod liver oil and 1.5 mg. alpha tocopherol	33	200	530	Normal	437
	34	200	536	Normal	418
	35	200	576	Normal	471
	36	200	510	Normal	437

their sides with their limbs rigidly outstretched. At autopsy the muscles appeared pale, shrunken and flabby. There was a marked decrease in creatine content of this tissue as contrasted with the normal. The guinea pigs receiving the two levels of wheat germ oil did not develop dystrophy and showed normal creatine values when sacrificed after the periods indicated. (One animal in each of the latter two groups developed a severe respiratory infection in the course of the experiment and was discarded.)

Because of the protective action afforded by wheat germ oil, an attempt was made to see whether the pure substance—alpha tocopherol—would act similarly. The yeast in the diet was increased from 5 to 10% with a corresponding 5% decrease in the cornstarch. This increase in yeast was deemed advisable in order to avoid a possible deficiency in the water soluble factor of Morgulis and co-workers.

The guinea pigs in the experiment were divided into two groups: (1) diet IV + 0.5 cc. cod liver oil daily; (2) diet IV + 1 cc. cod liver oil and 3 mg. of alpha tocopherol (dissolved in 8 drops of ethyl laurate) fed on alternate days. (This procedure was adopted as Cummings and Mattill had demonstrated that the autoxidation of cod liver oil was destructive to vitamin E.) The animals in each group received 5 cc. orange juice daily.

The results of these experiments are included in table 3. The eight guinea pigs in the control group exhibited dystrophy (20 to 67 days) and were practically moribund when sacrificed. The four animals receiving alpha tocopherol did not become dystrophic and were in good condition when sacrificed at 200 days and their muscle creatine values were found to be normal.

DISCUSSION

Guinea pigs maintained on a modified Madsen, McCay and Maynard diet were protected against nutritional muscular dystrophy and the muscle creatine was maintained within the normal range by the inclusion of: (1) 10% wheat germ in the diet (in the case of 5% wheat germ the clinical picture ap-

peared normal but the creatine values were below normal);
(2) 0.5 and 0.75 cc. wheat germ oil daily; (3) 1.5 mg. alpha
tocopherol daily (3 mg. on alternate days).

The guinea pigs receiving the purified diet averaged about
one-third less in weight than do animals in our stock colony
reared on commercial pellets and carrots. Our diet was in all
probability low in the "grass juice factor" of Kohler, Elveh-
jem and Hart ('38) and Kohler, Randle, Elvehjem and Hart
('39). Preliminary experiments conducted in our laboratory
would indicate that the yeast powder used in these experiments
carries the grass juice factor. In some cases respiratory in-
fection was noted. This has been the experience of other
workers using synthetic or purified diets (Madsen, McCay and
Maynard; Goettsch and Pappenheimer).

The suggestion by Mattill ('38, '39) as to the role of cod
liver oil in dystrophy-producing diets would offer an explana-
tion of our results.

SUMMARY

Wheat germ, wheat germ oil, and alpha tocopherol protect
against the nutritional muscular dystrophy that can be pro-
duced in guinea pigs reared on the Madsen, McCay and May-
nard cod liver oil-containing diet. It would, therefore, seem
apparent that when the vitamin B complex is adequately sup-
plied, vitamin E is the specific factor preventing nutritional
muscular dystrophy.

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FAT REQUIREMENTS OF THE GROWING CHICK [1]

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Little attention has been given to the fat requirements and metabolism of the chicken, despite the facts that the fattening of this species during growth is important and that the chicken puts a higher percentage of fat into the egg than the cow puts into milk. The object of the present study was to determine the effect on growth of as extensive a removal of fats and fat-like substances from an ordinary growing mash as is possible by means of fat solvents.

In a preliminary trial, birds fed an ordinary growing mash containing 4.0% fat grew to 885 gm. and those on a low fat ration, containing 0.025 to 0.074% fat grew to 769 gm. during the first 14 weeks of life. The fact that the low fat group did not show a marked nutritional failure led to a more carefully controlled experiment to determine whether the difference in growth response of the two groups was due to the differences in fat content of the rations or to a failure to supply adequate quantities of certain of the vitamins when the low fat ration was fed.

Since essentially the same procedure was used in the preliminary and in the final trial, only the latter will be described, with comments as to the important modifications which were introduced in an attempt to make the two rations more nearly alike, except for their fat content.

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[1] Journal series paper of the New Jersey Agricultural Experiment Station, Department of Agricultural Biochemistry. Presented before the Division of Biological Chemistry, American Chemical Society, Dallas, Texas, April 20, 1938.

Preparation of the ration. An all mash growing ration was used the percentage composition of which was as follows: ground yellow corn, 47; wheat middlings, 20; wheat bran, 15; meat scrap, 9; dried skimmed milk, 5; oyster shell, 3, and sodium chloride, 1.

To obtain the low fat ration the above mixture was extracted with diethyl ether.[2] Early in the experiment a Drake and Spies ('33) extractor was used and an extraction period of 150 hours reduced the fat content to 0.025%. Later the feed was extracted by allowing it to stand with diethyl ether for a 3-day period during which the ether was changed several times. This gave a product containing slightly less than 0.1% fat. The analyses were made by the Soxhlet method using a 20 gm. sample.

As a result of extraction, the low fat ration was dry and powdery as compared with the normal and was probably less palatable. The addition of 8% water to the extracted material gave it a consistency essentially the same as that of the normal ration. In order to replace in part the caloric value of the fat which had been removed and to maintain protein at essentially the same level as that of the normal ration, 5% sucrose was added to the low fat ration.

The fat content of the normal mash averaged 4.1% and that of the several batches of extracted ration 0.098%. The addition of ether-extracted yeast and sucrose further decreased the fat content to 0.088%.

Since the extraction procedure would remove fat-soluble vitamins and to some extent those of the water-soluble type, certain vitamin supplements were provided. To insure an adequate vitamin intake and to obviate the possibility of any difference in response due to differences in vitamin content, the same quantities of supplements were supplied to the normal as to the low fat group.

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[2] We desire to express our appreciation of the assistance rendered by Merck and Company, Rahway, New Jersey, in the extraction of a portion of the feed.

Vitamin A. Carotene was fed at the rate of 0.1 mg. per bird per day during the first 10 weeks of the experiment but during the remaining 4 weeks the quantity was doubled. On the basis of feed intake, each bird consumed an average of 550 I.U. (International Units) of carotene per 100 gm. of feed throughout the experiment. The quantity of carotene supplied was several times that reported by Record, Bethke and Wilder ('37), namely, 50 to 100 γ (80 to 160 I.U.) per 100 gm. of ration as the minimum for normal growth to 8 weeks of age.

The carotene was fed thrice weekly by capsule. It was dissolved in ether, the solution pipetted into capsules and the solvent removed in vacuo. The closed capsules were stored at 0°C. Under these conditions there was no loss of potency during 1 week.

Vitamin B factors. To insure an adequate supply of the vitamin B group of factors, 1% of yeast, extracted with cold ether, was incorporated in both the normal and low fat ration for the first 3 weeks of the experiment. For the remainder of the experimental period the level was increased to 2%. The average fat content of the several batches of extracted yeast was 0.224%, a quantity which would have a negligible effect on the percentage of this constituent in the whole ration.

Vitamin D. This factor was supplied by irradiation of the chicks with a quartz-Hg lamp for three 5-minute periods each week. The burner of the lamp was 65 cm. from the surface on which the chicks stood.

Other vitamins and essential factors. Extraction of the ration probably removed part or all of the antihemorrhagic factor, vitamin K. Certain birds in the first experiment showed subcutaneous hemorrhage but this condition was not observed during the second trial. Almquist and Stokstad ('36) have reported that the factor develops in moist fish meal or meat scrap and that it is formed in the intestinal tract of the chick on a vitamin K deficient diet. Thus the fact that water was added to the mash during the second trial and not during the first may account for the absence of vitamin K deficiency in the second experiment. Although the chicks were on raised

mesh floors, droppings could be consumed and it is possible that some of the factor may have been obtained from this source.

Vitamin E was probably removed or decreased markedly by the extraction procedure, although a rat assay of the ration was not made. No attempt was made to supply this factor because at the time this work was done it was available only in a fatty medium. Assuming that vitamin E was removed from the ration to a considerable extent by extraction, it could be concluded that this factor is not necessary for growth. This conclusion is in agreement with results reported by Sloan, Card and Adamstone ('37). These workers noted internal lesions which might be ascribed to vitamin E deficiency, but no internal abnormalities were observed in the present experiment.

Day-old White Leghorn chicks were fed a small quantity of the low fat ration for the first 3 days of life in order to deplete the fat reserves. They were then distributed according to weight into two groups of fifteen birds each. They were housed on wire mesh floors, at first in an electric brooder and later in a fattening battery. Individual weighings were made twice weekly.

RESULTS AND DISCUSSION

Of the fifteen birds started on each ration, only one of each group died. One bird was eliminated from the normal and two from the low fat group because they were stunted from the beginning of the experiment. One bird was discarded from the low fat group on account of a crooked beak.

The growth performance on the two rations is shown in table 1. Although the average weights of both males and females on the low fat ration were less than those on the normal at 14 weeks of age when the experiment was terminated, the differences were not marked as might be expected if an essential nutritional factor were lacking. Since only three birds were used in calculating the average weight of the males of the normal group for comparison with five of the low fat group,

the average weights of the females allow a better comparison. The difference between the average weights of the females of the two groups, 26 gm., is essentially the same as the difference between the weighted averages.

On account of the sex distribution, it was not possible to treat the data statistically according to sexes. However, when the growth responses of all of the birds of each group were considered, the probable error of the difference between the two means indicated that the difference in growth response is not significant.

TABLE 1

Growth response on normal and low fat rations

| | NORMAL RATIONS | | | LOW FAT RATIONS | | |
WEEKS	Males (3)	Females (10)	Weighted* average	Males (5)	Females (6)	Weighted average
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	36	36	36	36	36	36
2	100	101	100	99	97	98
4	162	179	170	179	178	179
6	277	303	290	327	301	314
8	456	462	459	508	414	461
10	653	609	631	692	556	624
12	912	820	866	917	778	847
14	1116	940	1028	1071	914	993

* Average weight of males plus average weight of females divided by 2.

Therefore the chick is able to grow at essentially a normal rate when ether-soluble substances have been removed from the ordinary all-mash type of ration to such an extent that the quantity of these substances remaining is of the order of 0.1%. The general appearance, conformation and gross internal anatomy of the low fat group was the same as that of the normal. However, it was noted that the fat depots of the birds receiving the low fat ration were nearly white, while the depots of the normal chicks were light yellow. Likewise, the bile and the livers appeared lighter in color in the chicks of the low fat group. These findings are attributed to the absence of pigments in the extracted ration and are in agreement with those of Palmer ('15) who showed that the feeding of pigment

free rations to hens resulted in the appearance of light-colored egg yolks, body fat, and blood serum.

An examination of the growth rates and the external and internal organs failed to show any evidence of vitamin A deficiency, and therefore it may be concluded that the growing chick is able to absorb and utilize sufficient carotene from a ration containing only a very small amount of fatty material. However, it should be noted that the quantity of carotene fed was of the order of five times that reported as the minimum requirement. It is possible that absorption of this factor is inefficient on a low fat ration and that a much larger quantity than the minimum is necessary in the digestive tract to assure adequate absorption. Experiments with hens in this laboratory (unpublished data) have shown that carotene is not well absorbed on a low fat ration.

TABLE 2

Iodine numbers

REGION	NORMAL DIET	LOW FAT DIET
Neck	76.3–76.6	58.4–59.6
Gizzard	76.5–76.8	58.5–59.9
Abdomen	74.7–76.9	60.1–60.3
Liver	90–113	93–112

Iodine numbers of the fatty acids of depot fat from the neck, gizzard and abdomen were determined by the method of Rosenmund and Kuhnhenn as modified by Yasuda ('31, '32) and are reported in table 2 along with those of liver fat. The values for the depot fat of the low fat group reveal a greater degree of saturation, in comparison with those of the normal. Thus the chicks on the low fat ration synthesized a more saturated body fat than that obtained from the feed by those on the normal ration.

In contrast with the iodine numbers for the depot fats, those for the liver fat of the two groups were essentially the same. This observation, and the fact that the liver fat was more unsaturated suggest that this fat is not of the depot or storage type, but that it is an indispensable component of the organ.

Whether there is a sufficient quantity of fatty acids held in the feed particles, even after prolonged extraction (Taylor and Nelson, '20) to meet any fat requirements of the chick can only be answered by the use of simplified rations of the type employed by Burr and Burr ('29) and others in studies with the rat.

SUMMARY

1. The extensive removal of substances soluble in diethyl ether from an ordinary poultry growing mash did not retard growth of chicks significantly up to 14 weeks of age, when care was taken to provide the vitamins removed by the extraction process.

2. Although dietary fat was reduced to a very low level (0.1% or less) crystalline carotene was utilized. However, the quantity of carotene fed was several times the minimum requirement.

3. The depot fat formed on the low fat ration was more saturated than that of chicks on the normal ration. On the other hand the liver fat of both groups showed essentially the same degree of saturation. The liver fat was less saturated than the depot fat.

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CYSTINE AND METHIONINE FOR GROWTH
AND LACTATION [1]

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Recent investigations have served to emphasize the importance of sulfur-containing amino acids in the processes of growth and lactation. Notable among these is a group (Jackson and Block, '32; Rose, '37 and '38; White and Beach, '37; Womack et al., '37) of studies which has established the indispensability of methionine for growth. These studies are now most commonly taken to mean (Rose, '37) that "cystine stimulates growth only when methionine is supplied in sub-optimal quantities."

Other investigations (Daggs and Lidfeldt, '38; Wright and Haag, '39) have dealt with the influence of cystine and methionine on lactation. That cystine and methionine promote lactation when fed with certain sulfur-deficient proteins like casein (Daggs and Lidfeldt, '38) or those from alfalfa (Wright and Haag, '39) appears to be well established. To what extent cystine and methionine serve to make these proteins biologically adequate in the conventional sense, and to what extent they may serve as specific dietary lactation stimulants, has not been so definitely determined.

Since the growth-promoting properties of arachin, the principal protein of the peanut, are enhanced by the addition of

methionine but not of cystine (White and Beach, '37; Baern-stein, '38) it appeared worthwhile to investigate commercial peanut meal as a convenient and inexpensive source of protein for rations deficient in their combined contents of cystine and methionine but which, like arachin, respond to methionine but not to cystine. The investigation of such rations should yield significant information concerning the conditions which govern the utilization of cystine and methionine in lactation.

TABLE 1

Composition of rations

INGREDIENTS	RATION NO.					
	121	122	123	124	125	126
	%	%	%	%	%	%
Peanut meal	20.0	20.0	20.0	36.0	36.0	36.0
Starch	53.0	52.8	52.8	37.0	36.8	36.8
Hydrogenated fat	19.0	19.0	19.0	19.0	19.0	19.0
Cod liver oil	1.0	1.0	1.0	1.0	1.0	1.0
Yeast	3.0	3.0	3.0	3.0	3.0	3.0
Salt mixture	4.0	4.0	4.0	4.0	4.0	4.0
l-cystine	...	0.2	...	...	0.2	...
dl-methionine	...	...	0.2	...	...	0.2

RATIONS

The rations were compounded as indicated in table 1. Solvent extracted commercial peanut meal furnished the principal source of protein. The peanut meal contained 40.00% crude protein and 0.30% organic sulfur.[3] The l-cystine [4] was prepared from hair. The dl-methionine was a synthetic preparation. The apparent digestibility of the dry matter in ration 121 was 92%; that of the crude protein, 81%.

[3] "Organic" sulfur is obtained by difference between total and "inorganic" sulfur. "Inorganic" sulfur is determined as follows: A 3 gm. sample is treated with 150 cc. of hot 2% trichloracetic acid, kept on a steam bath with occasional stirring for several hours, allowed to set overnight, filtered and washed. The "inorganic" sulfur is precipitated by treating the filtrate with an excess of BaCl$_2$.

[4] Furnished through the courtesy of Dr. C. S. Pease, Department of Chemistry.

PROCEDURE

The rations described in table 1 were fed to weaned young growing rats to determine growth-promoting properties. The members of any one pair or triplet were litter mates of the same sex.

Similar rations were fed to lactating females. The growth of litters of six nursing young was taken as an approximate measure of the lactation-promoting properties of the rations. An attempt was made to equalize food intakes within pairs or triplets. Numerous difficulties were encountered in food equalization because the size of our stock colony was not such as always to permit starting females within a pair or triplet on the same day.

RESULTS AND DISCUSSION

The results obtained by feeding the various rations to young growing rats are summarized in table 2. The addition of cystine, in harmony with other (Smuts and Marais, '38) recent work, did not improve the growth-promoting properties of the peanut meal proteins when fed at levels of 8 or 15%. The addition of methionine, however, caused a growth response in each of the six pairs or triplets in which it was fed. It therefore appears that, for the growth of rats, the mixed proteins of the peanut, like arachin (White and Beach, '37; Baernstein, '38) are improved by the addition of methionine but not of cystine.

Experiments in which lactating females were fed identical rations are summarized in table 3. There is a definite indication of a slight, though not necessarily significant, lactation response to cystine. This is in sharp contrast with similar experiments (Wright and Haag, '39) in which cystine strikingly improved the lactation promoting properties of alfalfa proteins. There appears to be a definitely significant lactation response to the addition of methionine, although here again this response is not as striking as that obtained when similar amounts of cystine were added to rations containing alfalfa

proteins.[5] At any rate, the magnitudes of the lactation responses obtained in these experiments are not such as to suggest that cystine and methionine play unique roles as lactation stimulants apart from making proteins biologically adequate in the conventional sense.

TABLE 2

Growth and food intake of weaned rats

PAIR OR TRIPLET	RATION NO.	DURATION OF EX- PERIMENT	INITIAL WEIGHT	FINAL WEIGHT	GAIN	TOTAL FOOD INTAKE	GAIN PER GRAM PEANUT PROTEIN
		weeks	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	121	6	43	63	20	229	1.09
	122		44	63	19	231	1.03
2	121	6	46	65	19	242	0.98
	122		44	60	16	243	0.82
3	121	6	37	51	14	172	1.02
	122		38	50	12	175	0.86
4	121	6	41	65	24	213	1.41
	123		41	72	31	217	1.79
5	121	6	45	58	13	193	0.84
	123		41	71	30	218	1.72
6	121	6	43	58	15	208	0.90
	123		37	69	32	229	1.75
7	124		40	96	56	291	1.28
	125	4	51	100	49	254	1.29
	126		45	126	81	292	1.85
8	124		38	95	57	265	1.43
	125	4	39	91	52	231	1.50
	126		39	110	71	246	1.92
9	124		43	83	40	207	1.29
	125	4	43	81	38	220	1.15
	126		48	109	61	222	1.83

We are keenly aware that the interpretation of our results in terms of lactation-promoting properties is complicated by the large losses in body weight of the lactating females as well as by the indirect method of measuring lactation. It seems, however, that for the present, the most plausible implication of our results is that the conditions which govern the utiliza-

[5] Unpublished data.

TABLE 3

Data on lactating females and nursing young

RAT NO.	RATION NO.	WEIGHT OF SIX YOUNG Days				WEIGHT OF FEMALES Days		21-DAY FEED INTAKE
		0	3	17	21	0	21	
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
2322	121	37	50	99	125	253	175	373
2324	122	39	55	107	125	272	202	365
2318	123	32	47	110	137	265	194	370
2319	121	38	53	109	132	288	204	380
2320	122	35	45	103	132	288	188	333
2323	123	38	50	114	144	274	181	331
2339	121	39	52	111	142	305	207	347
2390	122	37	56	112	134	266	200	347
2425	123	40	49	114	135	249	170	309
2333	121	37	48	107	140	320	224	332
2323	122	36	45	115	147	387	246	249
2421	123	41	54	120	160	249	165	305
2334	124	37	55	143	185	276	220	453
2300	125	37	48	145	185	290	286	452
2303	126	31	43	143	191	302	271	453
2309	124	42	63	165	221	255	240	548
2060	125	39	62	146	183	296	255	432
2305	126	39	60	185	239	333	290	548
2369	124	35	49	136	168	262	227	443
2368	125	35	45	139	165	292	238	443
2317	126	37	58	165	202	269	214	405
2315	124	34	41	124	145	281	240	431
2070	125	39	58	131	160	289	243	373
2310	126	33	47	129	160	273	228	411
2316	124	39	47	133	170	284	236	418
2308	125	36	59	150	176	328	280	418
2313	126	36	52	154	189	293	257	418
2391	124	40	54	132	165	262	222	432
2426	125	37	46	125	148	259	203	365
2397	126	38	52	142	183	265	224	443
2314	124	36	57	141	184	295	251	446
2399	125	34	47	119	153	276	239	360
2388	126	44	63	170	215	248	206	433
2417	124	35	50	130	170	246	216	431
2389	125	42	58	129	166	238	185	337
2400	126	33	47	160	204	292	250	431

tion of cystine and methionine are essentially similar for growth and for lactation.

SUMMARY AND CONCLUSIONS

1. Cystine did not significantly improve the growth-promoting properties of peanut meal protein but appears to have caused a slight improvement in lactation-promoting properties.

2. Methionine improved both growth- and lactation-promoting properties.

3. Our results indicate that cystine and methionine serve primarily to make sulfur deficient proteins nutritionally adequate rather than as unique lactation stimulants.

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THE EFFECT OF DIFFERENT LEVELS OF VITAMIN B₁ AND IRON ON THE RETENTION OF IRON AND THE FAT CONTENT OF NORMAL YOUNG RATS

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ONE FIGURE

The results of balance studies (Schlutz et al., '33; Oldham et al., '37) have indicated that a smaller amount of iron was retained when the vitamin B_1 intake was increased. An attempt was made recently (Schlutz et al., '38) to determine whether or not the lowered retention of iron was due to a specific effect of the vitamin by studying the retention of iron of an infant on a normal diet both with and without supplements of crystalline vitamin B_1. While the average retentions of iron in that study were also lower during the periods of high vitamin B_1 intake than during control periods, the differences were small and became less as the study progressed.

The aim of the present investigation was to determine whether or not the level of vitamin B_1 intake had any effect on the retention of iron in young rats. Animals, rather than infants were used to permit more crucial and better controlled experimental conditions. In general, the plan was to select groups of animals each containing three litter mates. One of each group served as a control and was analyzed for iron at the beginning of the experiment. The two remaining

received the same basal diet but varying amounts of crystalline vitamin B_1. At the end of the experiment their bodies were analyzed for iron and the retentions of this element determined by comparison with the iron content of the control.

Early in the study, it was noticed that the animals which received the higher vitamin B_1 intake were heavier than their litter mates on the lower vitamin intake. In order to determine the composition of this additional gain in weight, the bodies of the animals used in the second half of the study were analyzed for fat.

METHODS

Young rats, 21 to 22 days old, weaned from mothers fed the regular stock diet, were matched in groups of three with respect to litter, sex and weight. One of each group was sacrificed at the beginning of the experiment and analyzed. The others were placed in separate glass cages which were constructed similar to those described by Lang and Calvery ('37). The cages were kept under a wooden hood to prevent contamination. One side of the hood consisted of a large glass door with hinges to permit access to the cages. Ventilation was furnished by a number of holes in the hood which were covered with a double layer of cheese cloth.

Six series of experiments were carried out, six pairs of animals and six controls being used in each. Each series consisted of two experiments on three pairs of animals, carried out at different times but under the same conditions.

In series A the experimental period was 21 days; in series B, 14 days; in series C, 9 days and in series D, E and F, 7 days.

Two basal diets were used which for convenience, will be referred to as diet I and diet II. The composition of both is shown in table 1. In series A, B and C, diet I was given ad libitum and records were kept of the amount consumed. Half of the animals received a daily supplement of 3 mcg. of vitamin B_1,[1] while their litter mates received 14 mcg.

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[1] Crystalline thiamin hydrochloride was furnished by Merck & Co. The crystals were dissolved in 20% ethyl alcohol. One millimeter was equivalent to 40 mcg. of vitamin B_1.

daily. These supplements were given separately and were entirely consumed.

The daily iron intake in series A was augmented by the addition of 0.5 mg. of iron as ferric chloride, which made the total daily intake approximately 1.6 mg. In series B and C no additional iron was given and the daily iron intake was approximately 1 mg.

In series D, E and F, 5 gm. of diet II were given daily to all animals. This amount was completely consumed and was sufficient to give a gain in weight of slightly more than 2 gm.

TABLE 1

Composition of basal diets

DIET I	Per cent	DIET II [2]	Per cent
Purified vitamin free casein	20	Powdered whole milk [3]	45
Hydrogenated vegetable oil	17	Sucrose	42
Sucrose	57.5	Hydrogenated vegetable oil	10
Cod liver oil	3.0	Cod liver oil	3
Salt mixture [1]	2.5	0.04 mg. Mn and 0.10 mg. Cu	
2.0 cc. autoclaved rice polishings and 0.2 cc. lactoflavin $\leftrightharpoons$ 20 γ in solution as daily supplements		as daily supplements	

[1] Hubbell, Mendel and Wakeman salt mixture no. 351 was used.
[2] Diet II contained approximately the same amounts of protein, fat, carbohydrate, manganese and copper as diet I.
[3] Powdered milk in the form of Dryco was furnished by the Borden Company.

per day. Half of the animals in these series received no additional supplement of vitamin B_1 since the daily ration contained 9 mcg. of the vitamin.[2] Their litter mates received a daily supplement of 30 mcg. which made their daily intake 39 mcg.

The daily intakes of iron in series D, E and F were approximately 0.02 mg., 0.1 mg. and 0.3 mg., respectively. The additional iron in series E and F was again given as ferric chloride. It was mixed with a small amount of the daily ration and was readily consumed.

[2] The amount of vitamin B_1 in the daily ration was assayed by Dr. Elizabeth Knott, using rat growth technic.

The iron content of the basal diets and the supplements as well as that of both control and experimental animals was determined by analysis, using the thiocyanate method which has been described elsewhere (Schlutz et al., '33). The usual precautions were observed to prevent contamination. In all cases except those in which the fat content of the animal was also determined, the whole body less the gastrointestinal tract was ashed and an aliquot was analyzed. When fat was determined, the bodies were dried and ground and duplicate ashes were made of portions of the ground material.

Fat determinations were made according to the method of Holt, Courtney and Fales ('19).

<center>DISCUSSION OF RESULTS</center>

<center>*The effect of vitamin B_1 on iron retention*</center>

The average daily iron intakes, the average iron content of the controls and of the experimental animals, and the average daily retentions of iron are shown in table 2. Data for individual animals are included for series E only. The daily retentions of individual animals, and average retentions for the animals on the different levels of iron intake are shown in figure 1. Although the individual retentions vary, the averages consistently show that the different levels of vitamin B_1 intake had no appreciable effect upon the retention of iron. When average retentions for each series are considered, those of the animals on the higher vitamin B_1 intake agree very closely with those of the animals on the lower intake. The small differences which do occur are in favor of the higher vitamin intake in three of the six series. Therefore, it seems fairly certain that the amount of vitamin B_1 in the diet has no effect on the retention of iron in the rat under these conditions. This would suggest either that the lowered retention found in children was due to chance or that the infant is more sensitive than the rat to increased intakes of the vitamin. That the former is true seems improbable because of the number of times that a lowered iron retention was

TABLE 2

Intake and retention of iron of rats on different vitamin B_1 levels

	CONTROL ANIMALS		EXPERIMENTAL ANIMALS—VITAMIN B_1 3 MCG. PER DAY						EXPERIMENTAL ANIMALS—VITAMIN B_1 INTAKE 14 MCG PER DAY					
	Iron content		Iron content of experimental animal				Retention of iron		Iron content of experimental animal				Retention of iron	
	Total	Mg./gm.	Total	Mg./gm.	Weight at end	Iron intake Mg./day	Total	Mg./day	Total	Mg./gm.	Weight at end	Iron intake Mg./day	Total	Mg./day
Series A. Length of experiment 21 days														
Av.	1.739	0.043	4.770	0.053	90.24	1.578	3.031	0.144	4.840	0.049	99.51	1.736	3.101	0.148
Av.	1.788	0.042	4.566	0.052	88.38	1.534	2.779	0.132	4.536	0.051	90.18	1.487	2.749	0.131
Series B. Length of experiment 14 days														
Av.	1.419	0.038	3.554	0.051	69.34	0.950	2.135	0.153	3.017	0.045	67.24	0.901	1.598	0.114
Av.	1.353	0.036	3.178	0.048	65.80	0.950	1.825	0.130	3.107	0.044	71.60	0.973	1.754	0.125
Series C. Length of experiment 9 days														
Av.	1.696	0.036	3.056	0.051	59.44	1.044	1.360	0.151	2.989	0.048	61.85	1.123	1.292	0.143
Av.	1.696	0.036	2.993	0.047	64.52	0.985	1.297	0.144	2.975	0.048	62.62	0.933	1.279	0.142
VITAMIN B_1 INTAKE 9 MCG. PER DAY									*VITAMIN B_1 INTAKE 39 MCG. PER DAY*					
Series D. Length of experiment 7 days														
Av.	1.326	0.029	1.434	0.025	56.57	0.020	0.108	0.015	1.388	0.024	58.44	0.020	0.062	0.009
Av.	1.633	0.035	1.856	0.031	59.78	0.021	0.223	0.032	1.812	0.030	60.26	0.021	0.179	0.025
Series E. Length of experiment 7 days														
	1.799	0.038	2.414	0.039	61.82	0.119	0.615	0.088	2.488	0.040	62.27	0.119	0.689	0.098
	1.799	0.038	2.202	0.038	57.52	0.119	0.403	0.058	2.416	0.040	59.87	0.119	0.617	0.088
	1.823	0.039	2.463	0.043	57.33	0.119	0.640	0.091	2.314	0.039	59.20	0.119	0.491	0.070
Av.	1.807	0.039	2.360	0.040	58.89	0.119	0.553	0.079	2.406	0.040	60.45	0.119	0.599	0.085
	2.070	0.043	2.957	0.049	60.06	0.120	0.887	0.127	2.815	0.047	59.78	0.120	0.745	0.106
	2.070	0.043	2.594	0.044	59.15	0.120	0.524	0.075	2.680	0.046	57.99	0.120	0.610	0.087
	2.378	0.047	2.828	0.044	64.11	0.120	0.450	0.064	3.232	0.050	65.25	0.120	0.854	0.122
Av.	2.173	0.044	2.793	0.046	61.11	0.120	0.620	0.089	2.909	0.048	61.01	0.120	0.736	0.105
Series F. Length of experiment 7 days														
Av.	1.684	0.038	2.720	0.049	55.85	0.319	1.036	0.148	2.860	0.049	58.34	0.319	1.176	0.168
Av.	1.728	0.041	2.865	0.052	55.12	0.319	1.137	0.162	2.827	0.050	56.53	0.319	1.099	0.157

found in different infants when the vitamin B_1 intake was increased.

The iron intake necessary for optimal retention in the young rat

Little has been published concerning the optimum iron intake of the normal young rat. In order to obtain a true optimum intake for normal animals it is essential that the stores of iron be normal at the beginning of the experiment.
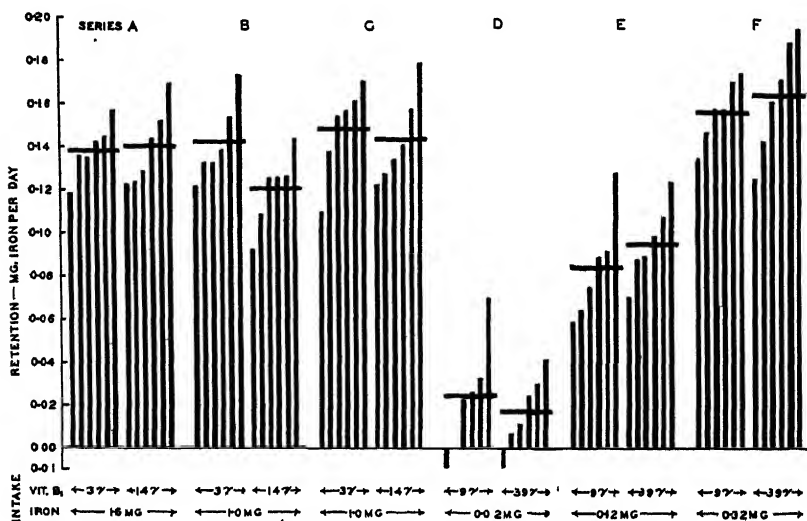
Fig. 1 Iron retentions in individual rats. The horizontal lines represent the average retentions of the groups.

This has not been the case in anemic animals which have invariably been used in studies of iron retention. In this study, the iron retentions of young rats with normal iron stores increased as the daily iron intake was increased from 0.02 mg., to 0.32 mg. (series D, E and F). When the daily intake was above 0.32 mg. (series A, B and C) no further increase was shown in the retentions. The average iron content of the different groups of animals showed the same trend as the average retentions. On daily intakes of 0.02 mg.,

0.12 mg. and 0.32 mg. the average iron contents of the animals were 0.028, 0.044 and 0.050 mg. per gram respectively. The average of all animals on higher intakes (1.0 to 1.6 mg.) was 0.049 mg. per gram. From these data it would seem that the optimal daily iron intake for the normal young rat is not more than 0.30 mg.

Both average daily retentions of iron and the average iron content of the bodies showed no significant variation in all series in which the daily intake was 0.32 mg. or more, regardless of whether the experimental period was 7, 14 or 21 days. This suggests that the rat stores iron at a fairly constant rate and that a 7-day experimental period is of sufficient length when the retention of iron is under consideration.

TABLE 3

Iron content of young rats

STUDY	NUMBER OF ANIMALS ANALYZED	AGE	WEIGHT	TOTAL IRON CONTENT	IRON CONTENT
		days	*gm.*	*mg.*	*mg./gm.*
Smythe and Miller ('29)	6	20	32.3	0.806	0.025
Present	36	21–22	44.4	1.678	0.038
Smythe and Miller ('29)	9	40	47.6	2.221	0.047
Present	12	42	92.1	4.678	0.053

It is interesting to compare the iron content of the animals in this study with the results obtained by Smythe and Miller ('29). The average weight and the average iron content of the rats used in both studies are shown in table 3. Although the animals used in this study were heavier and contained a larger amount of total iron than those of Smythe and Miller, the iron contents of the two groups of animals are quite comparable when calculated on a milligram per gram basis. This is especially true in the case of animals analyzed at approximately 40 days of age.

The influence of vitamin B_1 on the rate and composition of growth

It was noticed that the animals which received the higher vitamin B_1 intake (39 mcg.) in the first half of series D, E and

F were consistently heavier than their litter mates which received the lower vitamin intake (9 mcg.). The diets of the two groups of animals furnished the same number of calories and were identical in all respects other than the amount of crystalline vitamin B_1 given as a daily supplement. Fat determinations were made on the animals used in the second half of series D, E and F. The average fat content and that of individual animals are shown in table 4.

TABLE 4

Weight gain and fat content of paired animals on isocaloric intakes

ANIMALS RECEIVING 9 MCG. VITAMIN B_1 DAILY					ANIMALS RECEIVING 39 MCG. VITAMIN B_1 DAILY				
Initial weight	Final weight	Gain in weight	Dry weight	Total fat content	Initial weight	Final weight	Gain in weight	Dry weight	Total fat content
gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
48.0	66.70	18.70			49.0	69.40	20.40		
45.0	65.15	20.15			44.0	66.03	22.03		
47.0	65.38	18.38			47.0	66.35	19.35		
49.0	66.42	17.42	16.75	4.649	49.0	68.13	19.13	17.29	4.965
53.0	69.95	16.95	19.14	6.320	52.0	70.15	18.15	19.62	7.060
48.0	65.78	17.78	17.48	5.170	48.5	68.00	19.50	16.96	4.590
53.0	67.77	14.77			53.0	69.66	16.66		
48.5	65.00	16.50			49.0	65.82	16.82		
51.0	65.23	14.23			48.0	65.74	17.74		
53.0	67.56	14.56	18.46	5.604	52.0	66.35	14.35	18.53	5.920
50.0	66.25	16.25	18.13	5.635	50.0	63.80	13.80	18.80	6.603
54.0	69.89	15.89	19.46	6.059	56.5	71.03	14.53	21.83	8.280
50.0	64.75	14.75			50.0	64.98	14.98		
50.0	64.82	14.82			50.0	67.07	17.07		
46.0	60.97	14.97	17.15	5.956	46.0	63.18	17.18	18.59	7.165
45.5	58.52	13.02	16.38	5.013	45.0	61.82	16.82	17.38	5.690
47.0	61.86	14.86	17.18	5.542	47.0	62.17	15.17	16.85	4.850
46.0	62.47	16.47	17.14	5.180	46.0	63.27	17.27	17.86	5.741
Av. 49.1	65.25	16.14	17.73	5.518	49.0	66.28	17.28	18.37	6.086

During the experimental period of 7 days the animals receiving the higher vitamin B_1 intake gained an average of 1.1 gm. more than those on the lower intake. Statistical analysis [3] (Fisher, '36) indicated less than one chance in a hundred that such results could be due to pure chance.

[3] Statistical significance was determined by pairing the data and calculating t. Reference was then made to Fisher's table of t.

The average fat content of eighteen animals receiving 39 mcg. of vitamin B_1 daily was 0.56 gm. more than that of their litter mates receiving 9 mcg. Statistical analysis gave a probability of 0.08 and indicated that this difference might be due to chance. It is quite possible, however, that if fat determinations could have been made on all of the animals of these series, the difference in fat content in the two groups of animals would also have been statistically significant.

When consideration was given only to those animals of which the fat content was determined, those on the higher vitamin intake were found to have gained an average of 0.82 gm. more than their litter mates on lower intake. Fat (0.57 gm.) and water (0.18 gm.) accounted for 93% of the increase in gain.

There are many reports in the literature of superior growth on high levels of vitamin B_1 supply. In most cases the experimental animals have been compared with vitamin B_1 deficient animals and isocaloric diets have been used in very few of the studies. Whipple and Church ('36) found that in paired feeding experiments animals receiving vitamin B_1 were able to gain more weight than their litter mates on the same diet without the vitamin. Fat and water accounted for 94% of the increased gain. They ('37) also found that the respiratory quotient of B_1 deficient animals was low and could not be appreciably raised by the subcutaneous injection of glucose. Since the respiratory quotient of the isocaloric controls which had received the vitamin was higher and rose above unity under similar treatment, these investigators have suggested that vitamin B_1 is an essential metabolic constituent for the synthesis of fat in the animal body. They were comparing the weight and fat content of B_1 deficient animals with that of animals receiving the vitamin and consequently observed greater differences than were found in our animals, none of which received deficient diets.

It seems then, that the accelerating effect of vitamin B_1 on fat synthesis continues, although to a lesser extent, as amounts of the vitamin are ingested which greatly exceed those generally considered as adequate.

SUMMARY

Litter mate rats were given the same diets but varying amounts of iron and vitamin B_1 as thiamin hydrochloride. The iron contents of thirty-six groups of three litter mates each were determined and retentions were calculated using as a control the litter mate which had been sacrificed and analyzed for iron at the beginning of the experiment.

The level of vitamin B_1 intake did not influence the retention of iron at the levels studied.

The optimum daily iron intake for rats of this age as judged by the retention of iron and by the iron content of the bodies is not more than 0.30 mg. Intakes above this level failed to give an increase in storage.

An experimental period of 7 days was found to be of sufficient length to judge retention of iron.

When isocaloric intakes were given, animals receiving 39 mcg. of vitamin B_1 daily gained on an average 1.1 gm. per week more than their litter mates who were receiving 9 mcg. of the vitamin. This difference was found to be significant. Of the increased gain, 93% was found to be accounted for by increases in the fat and water content of the bodies.

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A SPECTROCHEMICAL STUDY OF THE NORMAL RANGES OF CONCENTRATION OF CERTAIN TRACE METALS IN BIOLOGICAL MATERIALS [1]

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ONE FIGURE

It has come to be recognized that certain metals, once believed to be foreign and dangerous to living material, or to be accidental, at most, in their occurrence therein, are regular and normal constituents of the tissues and excretions of animals and men. Conclusions as to the effects of these metals on health and disease must be based, therefore, on quantitative information as to the limits of their normal concentration in biological materials, as well as to the ranges of concentration which may give rise to signs of deficiency on the one hand or of excess on the other. In the pursuit of such information considerations of convenience, specificity, and accuracy have led us to use quantitative spectrochemical methods for the estimation of a series of trace metals in various biological materials under a variety of conditions. Our methods for the determination of lead (Cholak, '35 a, '35 b), and bismuth (Cholak, '37), were recently improved and were extended so as to make possible the simultaneous determination of a number of metals in a single small sample (Cholak and Story, '38).

The data presented herein are concerned largely with the concentrations of manganese, lead, tin, aluminum, copper, and

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silver which occurred in duplicates of the composite 24-hour food samples, in the excreta of apparently normal healthy human adults, and in the tissues of selected persons whose activities had given them no unusual or occupational exposure to these metals. A considerable volume of information on the lead content of individual food items, natural waters, and samples of soil from various portions of the earth's surface has accumulated since we recorded our earlier results and reviewed the work of other investigators (Kehoe et al., '33); this is presented herein. Reference to the numerous articles concerning the trace metals from other laboratories has of necessity been reserved for a separate review.

ANALYTICAL TECHNIQUE

Samples were prepared for analysis by procedures outlined previously (Cholak, '35 a, '35 b, '37; Cholak and Story, '38). Suitable quantities of solutions of the ashed materials, the inorganic salt compositions of which were made to conform to the standards by the method of excess (Cholak, '35 b, '37; Cholak and Story, '38), were placed in craters of purified graphite electrodes and their arc spectra were photographed while a five-step logarithmic sector was being rotated before the slit of the spectrograph (Cholak and Story, '38). The method of evaluating the spectral lines was similar to that of Strock ('36) except that opacities were substituted for densities in evaluating very weak lines (Cholak and Story, '38). The spectral region between 2600 A and 3500 A, in which the most persistent lines of a large number of the metals occur, was photographed. Although limited in application thus far to six metals, the method as applied to this region can be extended to include others, especially iron, thallium, cadmium and nickel. Zinc, on the other hand, cannot satisfactorily be dealt with simultaneously, since its most persistent lines at 3302 A and 3345 A lack sufficient sensitivity for the detection of the small amounts of zinc normally present and are also masked by the sodium line at 3302 A and the calcium line at 3345.5 A. The detection and determination of this metal are dependent,

therefore, on spectrograms made by a special technique in the extreme ultraviolet region (Zn line at 2138.5 A), which lacks suitably persistent lines of other metals and for which specially sensitized plates must be used. In the case of manganese, included since our latest publication (Cholak and Story, '38), the line at 2801 A was employed in conjunction with the chromium line at 2835 A as internal standard. The latter metal was added to the mixed internal standard, which then consisted of 5 mg. of bismuth, 100 mg. of cobalt and 10 mg. of chromium per 100 ml. of solution (Cholak and Story, '38).

The quantitative sensitivity of the technique used in these analyses was further increased by concentrating the solutions of the ashed materials to a point beyond that of previous practice (Cholak and Story, '38). The volumes of the final solutions of tissues and whole blood were adjusted so that each cubic centimeter was equivalent to 2 gm. of the original material, while in the case of blood plasma each cubic centimeter was equivalent to 3 ml. of original plasma. Such procedures permitted the detection of 0.005 mg. of metal per 100 gm. of fresh tissue or blood, and 0.003 mg. of metal per 100 ml. of plasma.

RESULTS

The results obtained on normal human tissues are recorded in table 1. With the exception of the tissues of one male whose occupational history was carefully checked, those examined were from females who had never been employed in industry. This choice of material has limited the number of available samples and has prevented the establishment of statistically stable mean values for the concentration of metals in the various tissues, but the arithmetical means are expected to fall near the eventual stabile values.

Table 2 gives the data on the concentrations of the metals in the urine of several widely scattered groups of normal men, including thirty-four Frenchmen, thirty Mexican Indians and thirty Americans. The results with respect to lead on a group of thirteen Germans are also shown. The urine (24-hour speci-

TABLE 1

The concentration levels of trace metals in normal human tissues (Americans)
(arithmetical mean values)

| TISSUE | MILLIGRAMS OF METAL PER 100 GM. OF WET TISSUE | | | | | |
	Mn	Pb	Sn	Al	Cu	Ag
Kidney	0.060	0.027	0.020	0.042	0.166	0.00
Heart	0.032	0.038	0.022	0.056	0.190	0.00
Brain	0.030	0.013	0.00	0.004	0.400	0.003
Liver	0.205	0.130	0.060	0.160	0.710	0.005
Spleen	0.022	0.030	0.022	0.130	0.085	0.00
Lung	0.022	0.028	0.045	5.94	0.110	0.004
Muscle	0.050 [1]	0.010	0.011	0.015	0.125	0.00
Long bone	0.300 [1]	1.88	0.080	0.500	1.190	0.00
Rib bone	0.170 [1]	0.470	0.050	0.240	0.410	0.01
Stomach	0.030 [1]	0.022	0.050	0.073	0.107	0.00
Intestines	0.035	0.023	0.016	0.087	0.110	0.002

[1] Results of single analyses.

TABLE 2

The concentrations of trace metals in normal urine expressed as the means,
their probable errors, and their standard deviations

| METAL | MILLIGRAMS OF METAL PER LITER OF URINE | | | | |
	Frenchmen	Mexicans	Americans	Germans	All groups
Mn		0.012±0.001 ±0.001	0.010±0. ? ±0. ?		0.01± ?
Pb	0.020±0.02 ±0.014	0.022±0.002 ±0.017	0.029±0.002 ±0.016	0.027±0.002 ±0.012	0.027±0.001 ±0.014
Sn	0.00±0.00 ±0.00	0.009±0.001 ±0.007	0.018±0.002 ±0.013		0.011±0.001 ±0.010
Al	0.114±0.006 ±0.048	0.054±0.004 ±0.031	0.052±0.003 ±0.022		0.078±0.002 ±0.032
Cu	0.036±0.003 ±0.026	0.039±0.003 ±0.024	0.028±0.002 ±0.019		0.034±0.002 ±0.024
Ag	0.00	0.00	0.00		0.00
No. of samples	34	30	30	13	94 [1]

[1] Total in case of lead 107, in case of manganese 60.

mens) of a healthy experimental subject was examined for 28 consecutive days and the results, expressed in milligrams of metal per liter of urine, are grouped in accordance with their frequencies of occurrence, in table 3, together with the calculated means, the probable errors of the means, and the standard deviations from the means.

Blood samples from thirty normal Americans and thirty normal Mexican Indians provided the results in table 4, in

TABLE 3

The concentrations of trace metals in successive daily urine samples of a normal adult American

MILLIGRAMS PER LITER	FREQUENCIES OF OCCURRENCE OF THE QUANTITIES INDICATED					
	Mn	Pb	Sn	Al	Cu	Ag
0.00 –0.009	28		7	1	2	28
0.010–0.019		1	17	1	5	
0.020–0.029		8	3	3	7	
0.030–0.039		14	1	5	3	
0.040–0.049		4		4	5	
0.050–0.059		1	.	4	1	
0.060–0.069				5	2	
0.070–0.079				1	2	
0.080–0.089				1		
0.090–0.099				2		
0.100–0.109						
0.110–0.119				1	1	
Number of samples	28	28	28	28	28	28
Mean	0.01	0.034	0.014	0.052	0.037	
Probable error	± ?	±0.001	±0.001	±0.003	±0.002	
Standard deviation	± ?	±0.008	±0.007	±0.025	±0.014	

which the mean concentrations of the metals in whole blood are shown. More detailed data obtained on twelve consecutive weekly samples of the blood of a normal subject appear in table 5. Blood samples of the American group were obtained in duplicate by dividing a 30 ml. sample of blood at the time of its withdrawal. One portion was used to determine the concentrations of the metals in the whole blood (table 4), while the other, after treatment with an anticoagulant (puri-

fied sodium citrate),[2] was centrifuged at once and the separated plasma was analyzed. The quantities of metals in the plasma of 100 gm. of whole blood were calculated in each case, and those in the formed elements were taken as the difference

TABLE 4

The concentrations of trace metals in normal blood

WHOLE BLOOD, MG. PER 100 GM.	Mn	Pb	Sn	Al	Cu	Ag
Mexicans:						
Mean	0.018	0.023	0.010	0.012	0.126	Trace
Probable error	±0.001	±0.0005	±0.0015	±0.001	±0.002	
Standard deviation	±0.010	±0.004	±0.012	±0.010	±0.018	
Americans:						
Mean	0.012	0.027	0.014	0.014	0.103	Trace
Probable error	±0.001	±0.0006	±0.0015	±0.002	±0.002	
Standard deviation	±0.006	±0.005	±0.012	±0.012	±0.013	
Whole group:						
Mean	0.015	0.025	0.012	0.013	0.114	Trace
Probable error	±0.001	±0.0004	±0.0009	±0.001	±0.002	
Standard deviation	±0.009	±0.005	±0.010	±0.011	±0.020	
DISTRIBUTION IN BLOOD (AMERICANS)[1]						
Whole blood:	.					
Mean	0.012	0.027	0.014	0.014	0.103	Trace
Probable error	±0.001	±0.0006	±0.0015	±0.002	±0.002	
Standard deviation	±0.006	±0.005	±0.012	±0.012	±0.013	
Plasma:						
Mean	0.004	0.0015	0.002	0.024	0.043	
Probable error	±0.0005	±0.0002	±0.0002	±0.002	±0.0015	
Standard deviation	±0.003	±0.0013	±0.0014	±0.012	±0.012	
Cells:						
Mean	0.008	0.024	0.011	0.003	0.059	
Probable error	±0.001	±0.0006	±0.001	±0.0003	±0.002	
Standard deviation	±0.006	±0.006	±0.008	±0.002	±0.016	

[1] Results based on 100 gm. of whole blood.

between plasma and whole blood. The calculated mean values for the whole blood, cells, and plasma of the group are listed in table 4. The histogram in figure 1 details the spread in the results obtained for the copper and lead content of the plasma.

[2] In order to check the possible precipitating effect of sodium citrate on the distribution of lead in the blood, a highly potent heparin preparation was used as the anticoagulant in a number of blood samples, with results similar to those in samples treated with sodium citrate.

TABLE 5

The concentration of trace metals in successive weekly blood samples of a normal adult American

MILLIGRAMS PER 100 GM.	FREQUENCIES OF OCCURRENCE OF THE QUANTITIES INDICATED						
	Mn	Pb	Sn	Al	Ag	MILLIGRAMS PER 100 GM.	Cu
0.000–0.0049			9	4	18	0.070–0.079	2
0.005–0.0099	5		3	5		0.080–0.089	
0.010–0.0149	4		4	3		0.090–0.099	4
0.015–0.0199	4		1	1		0.100–0.109	4
0.020–0.0249	4	1	1	1		0.110–0.119	2
0.025–0.0299		6		1		0.120–0.129	4
0.030–0.0349		1		1		0.130–0.139	2
0.035–0.0399	1	3		1			
0.040–0.0449				1			
Number of samples	18	11	18	18	18		18
Mean	0.016	0.030	0.008	0.015	0.00+		0.108
Probable error of mean	±0.001	±0.001	±0.001	±0.002	?		±0.003
Standard deviation	±0.008	±0.005	±0.006	±0.012	?		±0.018
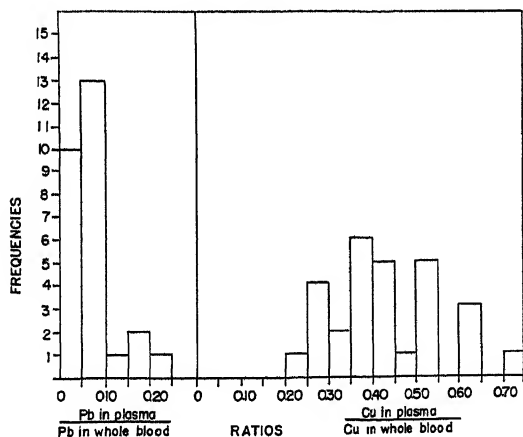
Fig. 1 Histogram showing the variation in the relationship between the plasma and the whole blood of normal human adults, with respect to their concentrations of lead and copper.

While the consecutive urine samples referred to in table 3 were being obtained, the corresponding feces and duplicate samples of the food and beverages taken by the subject were collected for analysis in 24-hour samples. The results are expressed in table 6 as mean daily intake and output.

Additional data concerning lead are given in tables 7 to 10.

TABLE 6

Comparison of the mean daily quantities of trace metals in successive samples of the feces and food of a normal adult American

| | MILLIGRAMS PER 24-HOUR SAMPLE | | | | | |
	Mn	Pb	Sn	Al	Cu	Ag
Food						
Mean	4.28	0.290	17.14	36.43	2.32	0.088
Probable error	±0.43	±0.027	± 1.30	± 7.90	±0.28	±0.010
Standard deviation	±3.38	±0.213	±10.17	±61.97	±2.21	±0.077
Feces						
Mean	3.69	0.320	22.88	41.92	1.96	0.058
Probable error	±0.30	±0.019	± 1.36	± 6.19	±0.18	±0.005
Standard.deviation	±2.30	±0.141	±10.28	±46.80	±1.33	±0.035

DISCUSSION

The concentrations of these metals in normal tissues, blood and urine are seen to be low, and, excluding copper and silver, are of the same order of magnitude. The concentration of copper is seen to be severalfold that of the other metals in practically all tissues, while in the case of silver, which is irregular in occurrence, only minute amounts are encountered. The pattern of the distribution of these metals in the human organism is roughly similar in that their highest concentrations generally occur, in decreasing order of magnitude, in bones, liver, kidney and spleen. The most striking exceptions are the concentration of copper in the brain, which ranks next to that in the liver, and the concentration of aluminum in the lung, which is higher than that in any other tissue, largely, no doubt, because of the inhalation of dust. Since aluminum is the most widely distributed metal on the earth's surface, inhaled dusts, especially from streets, may be expected to be rich in aluminum. This omnipresence of aluminum may exert

a serious influence upon the accuracy of determinations of this metal, since dust may be carried on the shoes and clothing of workers into the most ideal laboratory, the air of which is filtered. Our consistently low and substantially uniform results indicate that such contamination was kept at a minimum. In this connection it is to be remembered that the samples of blood plasma were concentrated one-third more highly than were the whole blood samples, and thus they gave somewhat higher results than the latter, on account of the increased analytical accuracy obtained thereby and not because of contamination.

Manganese, lead, aluminum and copper were regularly present in all samples of human tissue and urine, while tin was found in about 80% and silver in only 10% of the samples. Although all metals but silver were frequently present in blood, there were decided differences in the distributions of these metals between the cells and plasma. Thus, manganese, lead and tin were located principally in the cells, aluminum in the plasma, and copper fairly evenly divided between the two, the cells usually containing slightly larger quantities.

All six metals occurred regularly in the feces and in composite food samples, and in concentrations considerably greater than those found in the tissues and the urine. In the case of each metal, the actual quantities found in the series of 24-hour samples of feces were of the same order of magnitude as those in the 24-hour samples of food, a fact which, in view of the low concentrations in the tissues and the urine, not only demonstrates that each of these metals is eliminated almost completely by the alimentary tract, but also suggests that they are but scantily absorbed by the alimentary tissues.

The detailed sources from which these metals are derived require further elucidation. It is well known that certain foods are high in manganese and copper, and that tin, aluminum, and silver in the dietary are derived to a large degree from the use of food containers, cooking utensils, and tableware, made of these metals. Only in the case of lead, however, are our data sufficiently numerous and varied to provide a

TABLE 7

The natural lead content of soil, water, and vegetation

MATERIAL	SOURCE	RANGE	NUMBER OF SAMPLES
		p.p.m.	
Soil (dry)	Northwestern U.S.A.	7.6 – 15.7	10[1]
Soil (dry)	State of Mexico, Mexico	0.70 – 8.0	5
Soil (dry)	Yucatan	0.80–25.0	38
Soil (dry)	Sarawac	1.20 – 3.0	3
Soil (dry)	River bottom in Sarawac	4.8	1
Tea leaves (dry)	Ceylon	0.02	1
Fruit of tree (wet)	Yucatan	0.15 – 0.30	2
Foliage (wet)	,,	0.25 – 0.60	2
Bark (dry)	,,	0.04 – 0.40	112
Root (dry)	,,	0.05	1
Latex (wet)	,,	0.04 – 0.08	36
Bark (dry)	Sarawac	0.04 – 0.25	6
Latex (wet)	,,	0.02 – 0.04	4
Cocoa beans (dried husks)	Trinidad	0.40 – 1.60	14
Cocoa beans (dried nibs)	,,	0.03 – 0.35	13
Peas (green)	State of Mexico	0.03	1
Peas (green pods)	,, ,, ,,	0.05	1
Beans (green)	,, ,, ,,	0.15 – 0.26	2
Beans (green pods)	,, ,, ,,	0.04	1
Cherries (green)	,, ,, ,,	0.05	1
Apples (green, unsprayed)	,, ,, ,,	0.12	1
Pears (green, unsprayed)	,, ,, ,,	0.18	1
Corn (green stalk)	,, ,, ,,	0.05 – 0.11	3
Corn (green husk)	,, ,, ,,	0.26	1
Corn (green on cob)	,, ,, ,,	0.03 – 0.31	3
Wheat (green)	,, ,, ,,	0.27	1
Radishes (green)	,, ,, ,,	0.28	1
Sea water	Caribbean Sea	less than 0.02[2]	2
Sea water	Pacific Ocean	less than 0.02[2]	3
Sea water	Atlantic Ocean	0.003–0.005	2
River water	Sarawac	0.005	1
Pond water	,,	0.004–0.02	3
Well water	Mexico	0.009	1
Stream water	,,	0.009	1
Well water	U.S.A.	0.02 – 0.03	2

[1] Data of Jones and Hatch, Soil Science, vol. 44, p. 37 (1937).
[2] Limit of sensitivity of analytical method then in use.

fairly comprehensive picture of its origin in the diet. The wide distribution of lead is clearly indicated in tables 7 to 10. Its presence in the soil and in the vegetation growing therefrom, is shown in table 7. In our experience, as illustrated in these data, the natural lead content of vegetation rarely exceeds a small fraction of a part per million, but there is always some small quantity, whether the plant grows in the surface or is deeply rooted in the earth. There is a considerable variation among plants and in different parts of the same plant, with respect to lead concentration, but probably not to the extent indicated by the data, since certain of the samples could not

TABLE 8

The natural lead content of coral (Florida Keys)

LEAD IN PARTS PER MILLION	FREQUENCIES OF OCCURRENCE
0.00– 4.99	1
5.00– 9.99	7
10.00–14.99	11
15.00–19.99	4
20.00–24.99	1
25.00–29.99	
30.00	1
Total	25

TABLE 9

The lead content of bone (American). No occupational lead exposure

AGE IN YEARS	MILLIGRAMS Pb PER 100 GM. WET BONE	
	Rib	Femur
6	1.02	1.14
20?	1.11	
51	0.47	
64		0.80
70	0.39	3.59
75	0.60	2.89
95	0.56	1.36

be so handled as entirely to exclude the possibility of surface contamination with soil or atmospheric dust, although every effort was made to do so.

The occurrence of lead in natural waters is also indicated in table 7. The results on sea water bear a significant relationship to those obtained on a series of coral skeletons, as shown in table 8. The latter samples were obtained for us by R. L. Cary of Princeton University (whose assistance we gratefully acknowledge), from the Tortugas Laboratory of the Carnegie Institution, with such precautions in packing as would prevent surface contamination. These analyses were made because of speculation as to the possibility of the development of substantially lead-free soils on a coral island. Since it is apparent

that there is a selective absorption of lead by the coral skeleton, it is scarcely to be expected that a soil founded upon such material would support the growth of lead-free plants and animals.

TABLE 10

The lead content of various foods and drinks

ARTICLE	RANGE	NUMBER OF SAMPLES
	p.p.m.	
Wheat bread	0.02–0.16	8
Bran flakes	0.14–0.15	2
Crackers and pretzels	0.24–0.25	2
Spaghetti (prepared for eating)	0.06–0.21	2
Corn (dry)	0.24	1
Cornstarch	0.75–1.83	4
Corn syrup	0.21–0.49	2
Cocoa (20 brands)	0.4–11.5	25
Tea (dried leaves) Chinese	43.2	1
Cabbage	0.10–0.24	4
Fruits (raw and cooked)	traces–1.00	16
Beef liver (fresh)	0.29–0.40	2
Meat (cooked)	traces–0.63	9
Meat (ground, cooked)	0.15–0.18	3
Sausages (cooked)	0.16–1.60	4
Eggs (raw and cooked)	traces–0.12	6
Coffee (prepared for use)	0.01–0.03	2
Milk	0.02–0.04	3
Beer ·	0.01–0.09	21
Grape juice	0.04–0.40	7
Wine (domestic and imported)	0.05–1.51	10
Water	0.02–0.05	10
Water	0.37–0.92 [1]	3

[1] Obtained from building in which water was standing unused in pipes for some days.

As shown by the standard deviation of the mean value (table 6) the freely chosen daily diet of a normal healthy adult shows considerable variation in its lead content. It is not to be assumed that all such lead is of natural origin, although the results on commonly available foods and beverages, as listed in table 10, show that such is the origin of a considerable proportion of it. As we have pointed out elsewhere (Kehoe et al., '33), the increment of lead content above that which is a

natural constituent is due to contamination from a great variety of sources.

The essential equivalence of the mean daily intake and output of lead, as previously referred to in commenting on the results for the entire group of six metals, is worthy of special note in connection with the data of table 9, which fail to reveal any relationship between age and lead concentration in the skeletal tissues. These data are obviously too few to give direct and definite evidence against an indefinitely progressive accumulation of lead in the body in response to the customary regular intake of lead over a prolonged period, but they are presented because of the care taken against the inclusion of abnormal material among the samples.

SUMMARY

A quantitative spectrographic method of high sensitivity and precision has been employed for the simultaneous determination of lead, manganese, tin, aluminum, copper and silver in normal biological material.

1. Lead, manganese, copper and aluminum were present in all materials examined; tin was present in about 80% and silver in 10 to 20% of the samples.

2. The mean concentrations of these metals in a liter of normal urine has been found to be below 0.01 for manganese and 0.078, 0.034, 0.027, 0.011, and 0.00 mg. respectively for aluminum, copper, lead, tin and silver.

3. The mean concentrations of the metals in 100 gm. of normal whole blood are 0.114, 0.025, 0.015, 0.013, 0.012, and 0.00+ mg. respectively for copper, lead, manganese, aluminum, tin and silver.

4. Practically all of the manganese, lead and tin is contained in the formed elements of the blood; aluminum is found almost entirely in the plasma, while copper is almost evenly divided between the two, the formed elements usually containing a slightly higher concentration.

5. The concentrations of these metals in consecutive daily or weekly samples of urine and blood from the same individual are not constant but vary from sample to sample.

6. The daily output of this group of metals in the feces is practically equivalent to their daily intake in the diet.

7. The wide distribution of lead is indicated by data obtained on a large number of natural materials.

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CARBOHYDRATE VALUES OF FRUITS AND VEGETABLES [1]

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Convenience of diet prescription has necessitated the grouping of fruits and vegetables according to their carbohydrate content (Joslin, '37; Wilder, '37). The analytical data on which such classifications are based, however, are essentially from few sources (Atwater and Bryant, '06; Chatfield and Adams, '31; and Chatfield and McLaughlin, '31). The data for the latter two government publications are taken in part at least from Atwater and Bryant's tables.

Few clinicians realize that much of this data is only approximate or is inaccurate because it was obtained by the method of difference, i.e., protein, fat, moisture, ash, fiber were determined by direct methods; the difference between the sum of these per cent values and 100% was considered carbohydrate, and by inference carbohydrate available as fuel. Two types of investigations have demonstrated the inaccuracies of this method of analysis: first, McCance and his associates ('36) by direct methods investigated the English common foods and found the prevailing carbohydrate values too high, particularly for the low carbohydrate group. Results of direct determinations included in the government circulars of Chatfield and her co-workers point in the same direction. Our results obtained by an independent method closely agree with those of McCance. Second, when the problem was approached by determining the unavailable carbohydrates

(Williams and Olmsted, '35), we obtained results which were quite different from the accepted fiber values. In other words, we have found the values for available carbohydrate too high and for unavailable carbohydrate too low.

The clinical significance of this is evident when one considers the possible dietary latitude which this may allow. If the carbohydrate values are low, may not many vegetables be prescribed as vitamin carriers or to provide necessary roughage without regard to their carbohydrate content? The purpose of this communication is, (1) to present the data obtained from the sources indicated above and compare them with data secured by our own analytical methods, and, (2) to suggest a classification of vegetables and fruits based on, we believe, correct values of carbohydrate content.

GENERAL CONSIDERATION

Carbohydrates of vegetables and fruits may be broadly classified as available and unavailable sources of food energy.

The available group is composed of glucose, fructose, sucrose and starch, the latter two being converted by hydrolysis into simple sugars, fructose and glucose. Analytical methods must consider the extreme lability of fructose to alkali and heat. The unavailable group is composed of hemicelluloses, cellulose and lignin. Hemicelluloses constitute a large group of such related substances as pentosans, hexosans, pectins and uronic acids. One common denominator exits; about 70% of the hemicellulose group can be converted by chemical means into simple, non-fermentable sugars. In contrast to the hemicelluloses, celluose is a chemical entity and by hydrolysis can be converted quantitatively into glucose. Lignin, a material which gives strength to the cellulose structure of plants, is a hydrocarbon and is present in very minute quantities in edible plants.

Analytical methods for determining the unavailable group must take into account the extreme lability of the hemicellulose group. Certain fractions are soluble in alcohol, others in water and still others resist prolonged boiling in dilute alkali

and acid. In fact, the accepted method for determining crude fiber defines fiber as that portion which resists solution in boiling dilute alkali and acid. Williams and Olmsted ('35) discussed the errors of fiber determination. The criticism of the official method in use since 1870 is that it neither removes all nor accounts for this group of carbohydrates. This is an important consideration if carbohydrate-by-difference methods are used. With these considerations in mind we extended our method for unavailable carbohydrate determination to include a complete carbohydrate analysis on a single aliquot of one specimen of fruit or vegetable.

METHODS OF ANALYSIS

Space does not permit details of analytical technique; only an outline will be presented. At least three 1-pound samples of each fruit or vegetable were obtained from chain stores of St. Louis, weeks or months apart. They were prepared as is customary in the household by discarding refuse. The samples were then macerated or sliced and together with the expressed juice dried in a hot air oven at less than 60°C. They were then pulverized and stored in dark bottles. This enables one to obtain a homogenous sample and to analyze aliquots repeatedly. Futhermore, the carbohydrate content is raised by this procedure to an easily determinable level.

Aliquots of ½ to 2 gm. pulverized material were used for analysis. If a considerable portion of fat was present, e.g., peanuts, the fatty material was removed by ethyl ether extraction. The soluble sugars were then removed by 60% ethyl alcohol extraction at room temperature. This soluble fraction includes fructose, glucose, and sucrose, the latter of which was inverted by dilute acid below 70°C. The sugars were estimated by copper-iodometric reagents of Shaffer and Somogyi ('33) and the availability of this fraction confirmed by the fermentation technique of Somogyi ('27). The residue consisting of starch and unavailable carbohydrates was then submitted to pancreatic digestion in buffered-bile salt solution at pH 8 as

described by Williams and Olmsted ('35). Pancreatic diges-
tion has a twofold purpose: starch is converted into maltose;
and protein and fat essentially removed, the latter two com-
ponents no longer interfere with the unavailable carbohydrate
determination. The soluble fraction, maltose from starch, is
converted into glucose by weak acid hydrolysis, and then es-
timated by the copper-iodometric method. Only the fraction
fermentable by yeast was taken as representing the glucose
from starch. The non-fermentable portion was assumed to
represent soluble hemicellulose. The insoluble residue from
pancreatic digestion was treated by the methods of Williams
and Olmsted ('35).

Two precautions in the carbohydrate estimation deserve
emphasis: first, we have cleared all sugar solutions by the
mercuric sulfate precipitation method of West, Scharles and
Peterson ('29), an important detail when using modern sensi-
tive copper-iodometric sugar reagents; second, available car-
bohydrate is identified by biological methods, namely pan-
creatic digestion and yeast fermentation.

In order to test the reliability of our method we selected
ten representative common foods, and analyzed them for car-
bohydrate content by our technic. Moisture, ash, protein and
fat were determined by conventional methods. Between 90
and 100% of each air dried material was accounted for with
the exception of spinach, of which only 85% was accounted
for. If one remembers that leafy vegetables, such as spinach,
contain 90 to 95% moisture, it is not surprising that large
errors of recovery may be overlooked when the wet material is
analyzed, i.e., a 15% failure of recovery in a dried material
containing 90% solids becomes 1.5% if stated in terms of wet
weight. Table 1 presents our results. These results appear to
indicate reliability of the method.

DISCUSSION OF RESULTS

In table 2, we present a comparison of data obtained from
three sources: our own, that of McCance, and that of Chatfield.
The latter are divided into two groups; the first column pre-

senting the analyses Chatfield and associates obtained by direct chemical determination, and the second column the total carbohydrate as determined by difference by these workers. It is to be noted that in general there is much better agreement between the first three columns than between them and the determinations by difference. In general the latter values are considerably higher than those yielded by direct chemical determination.

TABLE 1

Composite analysis of certain vegetables [1] (air dried)

FRACTION	CARROTS 0.128 [2]	SWEET POTATO 0.318	PEAS, FRESH 0.250	POTATO, IRISH 0.212	CABBAGE 0.093	LETTUCE 0.047	TURNIP 0.087	SPINACH 0.083	CORN 0.247	FLOUR
	%	%	%	%	%	%	%	%	%	%
Moisture (105°C.)	12.7	5.9	6.2	6.2	21.6	12.0	15.6	4.7	9.1	12.6
Ash	7.5	2.3	4.0	3.3	7.3	8.0	8.6	19.9	3.3	0.4
Nitrogen × 6.25	11.9	4.8	28.3	7.3	15.2	16.0	15.2	28.8	16.2	11.3
Ether-soluble	1.3	2.3	11.5	0.3	0.3	3.2	1.9	9.1	4.1	1.1
Available carbohydrate	47.5	75.0	33.1	77.2	26.9	30.5	40.9	7.9	60.0	70.6
Water-soluble hemicellulose	2.2	3.0	2.0	0.0	5.1	6.9	1.2	5.2	0.7	0.0
Cellulose	8.2	2.0	4.4	1.7	8.8	8.1	5.5	4.6	4.3	2.0
Non-water-soluble hemicellulose	6.5	1.0	7.0	1.0	5.2	5.8	4.5	4.7	2.9	0.0
Total (air dried)	97.8	96.3	96.5	97.0	90.4	90.5	93.4	84.9	100.6	98.0
Total (wet weight)	98.9	98.6	98.9	100.2	98.3	99.6	100.9	98.7	100.1	98.0

[1] Based on three specimens.
[2] Factor for conversion to wet weight.

The data presented by Chatfield are very striking with respect to the wide variation of carbohydrate content found between samples. Our data are based on three samples collected at various times of the year in St. Louis. In certain vegetables the results were quite consistent while in others there was great variation. Those which appear to vary widely are apples, corn, spinach, brussels sprouts, and beans. Others show very close agreement. Certainly not all food samples

TABLE 2

A comparison of different analyses for "available carbohydrate"

FRUIT	AUTHORS'	MCCANCE	CHATFIELD (CIRC. 146 AND 50) Invert sugar plus starch	CHATFIELD (CIRC. 146 AND 50) Total carb. less fiber
	%	%	%	%
Apples	11.5	11.7	11.1	13.9
Apricots	...	6.7	10.4	12.3
Apricots, dried	46.7	43.4	...	62.5[3]
Banana	...	19.2	19.2	22.4
Blackberries	...	6.4	6.1	7.8
Blueberries	...	...	9.7	13.9
Cherries	...	11.9	11.6	14.5
Cranberries	...	3.5	4.2	9.9
Currants	...	5.5[4]	5.7	9.5
Figs	...	9.5[5]	16.2	17.9
Gooseberries	...	3.4	4.2	7.6
Grapefruit	...	5.3	6.5	9.8
Grapefruit juice	...	...	6.7	...
Grapes	...	15.5	11.5	14.4
Grape juice	...	...	16.8	...
Lemon juice	...	1.6	2.3	...
Lime juice	...	...	0.3	7.8
Muskmelon	...	5.3	5.4	5.1
Nectarine	...	...	11.8	16.6
Orange	11.4	12.4	8.8	10.6
Orange juice	...	8.5	9.0	...
Peach	...	9.4	8.8	11.4
Pear	...	9.1	8.9	14.4
Pineapple	...	10.4	11.9	13.3
Plum	...	11.6	8.3	12.4
Prunes, fresh	...	9.6	13.3	21.3
Prunes, dried	50.6	40.3	...	73.3[3]
Raspberries	...	5.6	7.9	12.1
Rhubarb	...	1.0	0.4	3.1
Strawberries	...	6.2	5.3	6.9
Watermelon	...	...	6.0	6.3

VEGETABLES	AUTHORS'	MCCANCE	CHATFIELD (CIRC. 146 AND 50) Invert sugar plus starch	CHATFIELD (CIRC. 146 AND 50) Total carb. less fiber
	%	%	%	%
Asparagus	...	1.1[2]	1.4	3.2
Beans, string	5.8	2.9	2.6	6.3
Beets, red	7.3	9.9[2]	...	8.7
Broccoli	1.1	1.1[2]	1.9[1]	4.2
Brussel sprouts	2.6	1.7[2]	...	7.6
Cabbage	2.5	3.5	3.5	4.3
Carrots	6.0	5.4	7.5	8.2
Cauliflower	1.0	1.2[2]	2.6[1]	4.0
Celery	...	1.3	1.3	3.0
Corn, sweet	14.8	...	18.9	19.7
Cucumber	...	1.8	2.6	2.2
Dandelion greens	...	...	0.9	7.0
Egg plant	...	...	...	4.6
Endive	...	...	...	3.2
Kale	...	3.1	1.4	6.0
Kohlrabi	...	1.0	2.2	5.6
Lettuce	1.4	...	1.6	2.3
Mustard greens	...	1.8	0.4	3.2
Onions	...	5.2	7.2	9.5
Parsnips	...	11.3	11.9	16.0
Peas	8.3	10.6	11.4	15.5
Potato, Irish	16.4	20.8	15.6	18.7
Pumpkin	...	3.4	5.1	6.0
Radish	...	2.8	3.4[1]	3.5
Rutabaga	...	...	6.7	7.6
Spinach	0.7	1.4[2]	0.3[1]	2.6
Squash, summer	...	...	1.0	3.4
Squash, winter	...	...	4.9	7.3
Sweet potato	23.7	20.1[2]	25.6	26.9
Tomato	1.6	2.8	2.8	3.4
Turnip	3.5	3.8	4.6[1]	6.0
Turnip tops	...	...	...	4.2
Yams	...	...	18.7	23.3

[1] Only one or two analyses.   [2] Boiled.   [3] Bulletin 28.

[4] Average of black, red and white.   [5] Green.

vary widely, and we believe that some of the variations of analysis can be explained on the basis of the analytical method.

Table 3 presents data on the unavailable carbohydrates of twenty vegetables and fruits. For comparison, the first column gives the data of the government bulletins expressed as "fiber." It is readily seen that the sum of the hemicellulose plus cellulose is usually twice as great as the fiber value. We know of no data where these unavailable carbohydrates have been directly determined.

TABLE 3

Comparison of fiber and indigestible residue

MATERIAL	FIBER (CIRCU-LARS 50 AND 146)	HEMI-CELLU-LOSE	CELLU-LOSE	MATERIAL	FIBER (CIRCU-LARS 50 AND 146)	HEMI-CELLU-LOSE	CELLU-LOSE
	%	%	%		%	%	%
Beans, snap	1.4	2.95	0.5	Potato	0.4	0.3	0.4
Beets	0.9	0.8	0.9	Spinach	0.6	0.8	0.4
Broccoli	1.3	0.9	0.9	Sweet potato	1.1	1.4	0.6
Brussel sprouts	1.3	1.5	1.1	Tomato	0.6	0.3	0.2
Cabbage	1.0	1.0	0.8	Turnip	1.1	0.4	0.5
Cauliflower	0.9	0.6	0.7	Apples	1.0	0.7	0.3
Carrots	1.1	1.7	1.0	Apricots (dried)		3.5	2.2
Corn	0.9	0.9	1.1	Orange	0.6	0.3	0.3
Lettuce	0.6	0.6	0.4	Prunes		2.0	0.4
Peas	2.2	2.2	1.1	Peanuts	2.5	3.8	2.4

Classifications of fruits and vegetables

For simplicity of diet calculations the fruits and vegetables have been placed in groups and a single value of carbohydrate assigned to the group. The generally accepted classification of this kind is that of Adams and Chatfield ('35). They have set these values: group I, 3% (range 1.5 to 4.4); group II, 6% (range 4.5 to 7.4); group III, 9% (range 7.5 to 10.4); group IV, 12% (range 10.5 to 13.4); group V, 15% (13.5 to 16.4); group VI, 18% (range 16.5 to 19.4). Diabetic manuals prepared for patients such as those of Joslin ('37 and Wilder ('37) have accepted in general this classification, and text-

books of dietetics (Stern, '36; Sherman, '37; Rose, '37; Mc-Lester, '39; Bridges, '35) use the data for total carbohydrate as given in circulars 146 and 50. In their original article Adams and Chatfield state, ''The figures on which the new classification is based are those for nitrogen-free extract, that is, total carbohydrate excluding fiber.'' It would appear that there has been a general acceptance of the carbohydrate values of fruits and vegetables as determined by difference. No real defense can be raised for this method of carbohydrate analysis.

Table 4 presents our classification of the common vegetables and fruits according to their carbohydrate content.

TABLE 4

Classification of fruits and vegetables according to their carbohydrate content

1% (0.3–2.0)	*3%* (2.1–4.0)	*5%* (4.1–6.0)
Asparagus	Beans, snap (young)	Beans, snap (medium)
Broccoli	Brussel sprouts [1]	Blackberry
Cauliflower	Cabbage	Carrot
Celery	Cranberry	Currant
Cucumber [1]	Egg plant	Muskmelon
Greens	Gooseberry	Pumpkin
Dandelion [1]	Lemon juice	Strawberry
Kale	Radish [1]	Squash, winter
Mustard [1]	Tomato	Watermelon
Endive [1]	Turnip [1]	
Spinach		
Lettuce		
Rhubarb		
Squash, summer		

7% (6.1–8.0)	*9%* (8.1–10.0)	*11%* (10.1–12.0)
Beet, red	Apricots	Apple
Grapefruit	Blueberry	Cherry
Onion	Orange	Corn, sweet (young)
Raspberry	Orange juice	Fig
Rutabaga	Peach	Grape
	Pear	Nectarine
	Plum	Parsnip
		Pea
		Pineapple

[1] Insufficient analyses.

The 1% group corresponds to what Joslin calls 1 to 3% and what other authors classify as 3 to 5%. There is one very curious analysis which has crept into the literature, namely that of dandelion greens. All of the above-mentioned authors except Wilder and Joslin have classified this food as having a value of 2 to 11% carbohydrate. The green leafy vegetables that we have analyzed have not contained more than 1 to 2% carbohydrate. Our 3% group is classified by others as 3 to 9%; our 5% as 6 to 12%; our 9% as 12%.

What justifications have we for proposing these changes in classifications? Our argument is based upon the analyses reported by three different sets of observers. There is, first, the direct analysis recorded by Adams and Chatfield (circulars 146 and 50) which, as table 2 shows, corresponds closely to our own results. Second, we have the direct analyses of McCance and his associates on English vegetables and fruits, whose data are unique with respect to thoroughness and accuracy, and we believe, in the absence of systematic analyses of American fruits and vegetables, their data might well be adopted. Attention is called to the fact that most of the root and leafy vegetables contain only sugar which is easily lost when cooked in water. McCance found, for instance, that by boiling carrots 1 hour, 70% of the sugar was lost in the cooking water. The English authors make the point that since some of these foods are eaten only when cooked, the carbohydrate value should be stated for the cooked food.

In reviewing the tables of bulletins 146 and 50 for data obtained by direct analysis we find that of certain commonly-used vegetables and fruits there are none, or at the most, only two samples analyzed. The following is a partial list:

Beets	Dandelion Greens	Okra
Broccoli	Egg Plant	Radishes
Brussels Sprouts	Endive	Spinach
Cherries	Kohlrabi	Turnips
Cucumbers	Lima Beans	Turnip Tops

DISCUSSION

If it is granted that the carbohydrate values we propose are correct, the question might be raised as to why physicians have not become aware of the inaccuracy of the present values, especially since these foods are the chief foods of severe diabetics. Our answer would be that the amount of carbohydrate is not very great, whatever the standard of carbohydrate value calculated as available from these foods; and furthermore, the higher the amount of carbohydrate in a food the less the difference between the values assigned by ourselves and those generally accepted. This difference might only be apparent when a higher group is substituted for a lower one and even in this circumstance, the difference would not be very great and only evident in a very severe diabetic. Since we have had insulin, there has been a progressive tendency to prescribe higher carbohydrate diets and to use foods which have a higher value of carbohydrate.

The real worth of a realization of the true carbohydrate value of vegetables and fruits lies in the knowledge that physicians may prescribe the vegetables in the first two groups almost without regard to carbohydrate content. If we recall that these foods are very high in vitamin values, there is a good deal of virtue in feeding them without consideration of their carbohydrate contents.

It also seems apparent that the time is opportune for a complete and thorough re-analysis of the carbohydrate values of vegetables and fruits, not only for the available carbohydrate but also for the hemicellulose and cellulose. The importance of this problem and its demands with respect to laboratory facilities and funds doubtless place an elaborate project dealing with it beyond any single laboratory. Cooperative efforts in dealing with this problem would seem to be in order.

CONCLUSION

An outline of a method of analysis for sugar, starch, hemicellulose and cellulose of vegetables and fruits is presented.

. Representative carbohydrate values of twenty foods analyzed by this method are presented.

The accepted values for available carbohydrate are too high because they have been based on the "total carbohydrate by difference."

Fiber values are only a variable fraction of the true determined values for hemicellulose plus cellulose.

It is suggested that the time is opportune for a complete re-analysis of American vegetables and fruits for both their available and unavailable carbohydrates.

Comparison of data obtained by direct methods of analyses prompted a re-classification of low carbohydrate vegetables and fruits.

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ON THE DIFFERENCE BETWEEN THIAMIN DEFICIENCY IN THE RAT AND DEFICIENCIES OF THE OTHER MEMBERS OF THE VITAMIN B COMPLEX

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At the present time four crystalline members of the vitamin B complex are known, namely thiamin, nicotinic acid, riboflavin, and vitamin B_6 (factor 1). Nicotinic acid has not been clearly demonstrated to be necessary for the rat although indispensable to the dog (Elvehjem et al., '37), monkey (Harris, '38), pig (Chick et al., '38 a; Hughes, '38) and man (Fouts et al., '36; Smith et al., '37; Spies et al., '38). Undoubtedly nicotinic acid must play a role in rat metabolism since it forms a part of the enzyme system involving hydrogen transfer in metabolism of carbohydrates. Either, like vitamin C, it can be synthesized by the rat or its stores are so well conserved as to render exhaustion from the rat body tissues difficult or impossible. Possibly traces in the diet render this more difficult.

In addition to thiamin, riboflavin, and vitamin B_6 (factor 1), one or more factors not yet isolated in crystalline form are necessary for the rat. Factor 2 (Lepkovsky et al., '36), factor W (Elvehjem et al., '36), yeast filtrate factor (Edgar and Macrae, '37), or Filtratwachstumsfaktor B_w (Kringstad and Lunde, '39) will apparently complete the growth requirements of the rat for the vitamin B complex. These factors

have much in common and may or may not be identical. From its method of preparation, the concentrate used in the present work resembles most closely factor 2. Since it supported normal growth, it must have contained the other factor or factors necessary for the growth of the rat.

In the absence of any one of these four essential vitamins, normal health, growth, and life are impossible in the rat. Deficiencies caused by withholding any one of these essential vitamins were studied in groups of rats kept under identical experimental conditions during the same period of time. Thus, the only variable was the absence from the rat's diet of a single known essential member of the vitamin B complex. Some rats of each deficient group were given the missing vitamin at appropriate times to determine the ability of the rats to grow after being subjected to a severe vitamin deficiency.

EXPERIMENTAL

Female rats 21 days old weighing 40 to 50 gm. were weaned from normal stock mothers receiving a diet consisting of whole red wheat ground 57.5, wheat germ 10, commercial casein 15, whole milk powder 10, sodium chloride 1, calcium carbonate 1.5, and whole butter 5%, respectively. They were placed in individual cages with wire screen bottoms and fed a basal diet and water ad libitum. The percentage composition of basal diet was sucrose 59, extracted casein 27, primex 8, cod liver oil 2, and salt mixture no. 185 (McCollum and Simmonds, '18) 4. The preparation of the ingredients has already been described (Dimick and Schreffler, '39).

Four groups of rats, seven in each group, were fed the vitamin supplements as detailed in table 1. These supplements were fed daily except Sunday.

The thiamin was the synthetic product.[1] The riboflavin was a crystalline product prepared from whey. The crystalline vitamin B_6 was prepared from rice bran extract according to the method of Lepkovsky ('38). The vitamin B_6 eluate was

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[1] Sold by Merck & Company.

the crude extract described by Lepkovsky ('38) as the starting material for the preparation of crystalline vitamin B_6. It was previously tested to insure its freedom from the other known factors of the vitamin B complex. The factor 2 concentrate was a liver preparation as described by Dimick and Schreffler ('39).

The dosage levels were chosen for maximum growth and were maintained throughout. The level of vitamin B_6 eluate was selected to supply 25 micrograms of crystalline vitamin B_6; this was determined by separate assay. The factor 2 concentrate used was previously assayed to insure its adequacy for normal growth and its freedom from thiamin, riboflavin and vitamin B_6.

TABLE 1

GROUP	DEFICIENCY	SUPPLEMENTS			
		Thiamin	Riboflavin	Vitamin B_6 Eluate $\cong$ 25 mcg.	Factor 2
		mcg.	*mcg.*	*cc.*	*cc.*
1	Thiamin		25	0.05	0.10
2	Riboflavin	25		0.05	0.10
3	Vitamin B_6 (factor 1)	25	25		0.10
4	Factor 2	25	25	0.05	

Weekly weights and daily notations of occurrence of symptoms were made. When the rats were moribund or in a severely deficient condition, the missing vitamin was fed in crystalline form when available. This was possible with all the vitamins except factor 2. The response in growth and the improvement of the deficiency condition were noted. During the curative period, the rats were weighed daily and changes in condition noted.

RESULTS AND DISCUSSION

An inspection of figure 1 shows that the growth response of the rats to the addition of each of the four missing factors is approximately the same. In all cases excellent growth was obtained. Deficiency symptoms in all cases were promptly cleared up.

Thiamin deficiency differs from the other three deficiencies by its acute nature during depletion. After a moderate initial growth, the rats immediately started losing weight rapidly. With the other three deficiencies, after an initial growth, the depletion is characterized by a more or less prolonged period of stationary weight or a very slowth growth. This plateau period in the growth curves of the rats during the depletion period of all deficiencies except thiamin has been insufficiently emphasized.

It is improbable that nerve degeneration occurs in thiamin deficiency (Engel and Phillips, '38) possibly because of the acute nature of the deficiency. On the other hand, it is known
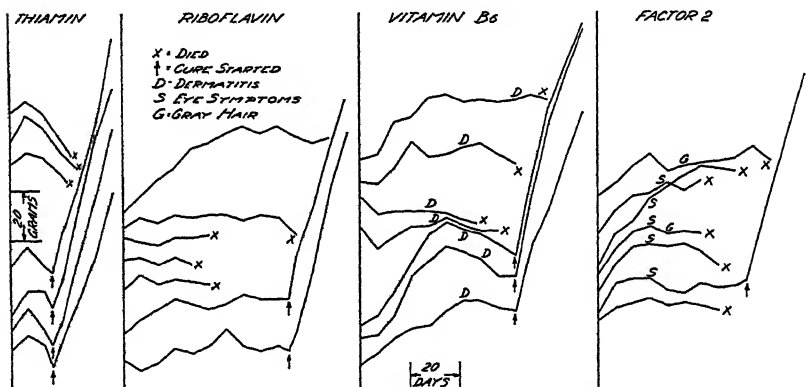
Fig. 1　Shows the individual curves of four groups of female rats started on the basal sugar diet and various combinations of the factors of the vitamin B complex. These factors were fed in amounts as indicated: 25 mcg. thiamin, 25 mcg. riboflavin, 0.05 cc. vitamin B_6 eluate equivalent to 25 mcg. vitamin B_6 (factor 1), and 0.1 cc. factor 2 concentrate. In each group one factor is omitted during the depletion period and added at the arrow.

The thiamin group shows the depletion period of rats deficient in thiamin. Twenty-five micrograms of thiamin were added daily to the diet of four members of the group as indicated by the arrow.

Riboflavin group shows the depletion period of rats deficient in riboflavin. Twenty-five micrograms of riboflavin were added at the arrow.

Vitamin B_6 group shows the depletion period of rats deficient in vitamin B_6 (factor 1). Twenty-five micrograms of crystalline vitamin B_6 were added at the arrow.

Factor 2 group shows the depletion period of rats deficient in factor 2. One-tenth cubic centimeter factor 2 concentrate was added at the arrow.

that riboflavin deficiency results in nerve degeneration (Phillips and Engel, '38) probably because of the chronic nature of this deficiency. Nerve degeneration also occurs in factor 2 (filtrate factor) deficiency in the pig (Chick et al., '38 b). This deficiency, like that of riboflavin, is chronic in nature.

These results may have a clinical bearing since they emphasize the acute nature of thiamin deficiency as compared with the chronic nature of the deficiencies of the other vitamins. Clinically nutritional deficiencies are generally multiple in nature (Spies et al., '39), but marked responses are often obtained with the administration of thiamin only (Williams and Spies, '38). This is understandable in the light of the acute nature of thiamin deficiency. It is to be expected that with continued administration of thiamin alone with no other dietary change, one of the more chronic deficiencies might develop.

CONCLUSIONS

Thiamin deficiency is characterized by the absence of a plateau period in the growth curve of the deficient rats during the depletion period. A plateau period, however, characterizes the depletion periods of deficiencies of riboflavin, vitamin B_6 and factor 2 (rat filtrate factor).

The implication of this difference is briefly discussed.

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THE ESTIMATION OF METHANE PRODUCTION BY CATTLE [1]

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ONE FIGURE

In connection with measures of nutritive values of foods and nutritive requirements of animals of different species, a factor of some importance is the fermentative activity normally present within the ruminant alimentary tract, especially as this bacterial metabolism involves losses of energy value in the forms of heat and methane, and gains in both protein and energy values by virtue of the capacity of the bacteria to render digestible non-nitrogenous products otherwise not digestible by the host, and to synthesize protein from non-protein nitrogen.

In the course of the researches of this laboratory, since 1902, many measurements of methane production by cattle have been made, always by the same procedure, in which the methane in an accurately measured aliquot of the outgoing air from a respiration calorimeter was burned to carbon dioxide and water; and the variations observed in these measurements indicate that determinations of methane production, by cattle, of an accuracy sufficient for research

611

purposes, must be made by measurement rather than computation.

For conventional, routine purposes, however, in the absence of a respiration chamber or calorimeter, it is sometimes desirable to compute the approximate production of methane. A statistical study has therefore been made of the relationship between methane production and various ration characteristics, with the object of deriving a formula by means of which methane production can be computed with an accuracy sufficient for the less critical purposes for which such
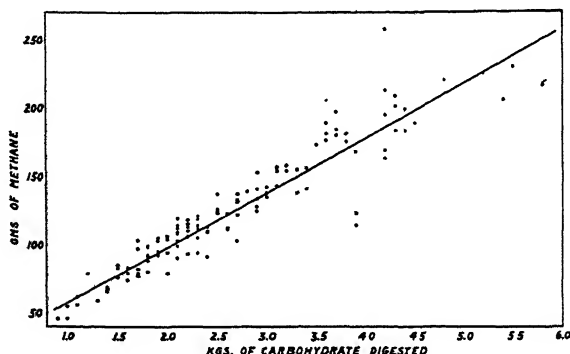
Fig. 1   Methane production in relation to digested carbohydrate.

a value might be used, such as, for instance, in the computation of metabolizable energy values of feeding stuffs for cattle.

After considering a number of such relationships the most significant one found was that between methane production and quantity of carbohydrate digested.

The available methane determinations of this laboratory were therefore plotted against the digested carbohydrate of the rations from which the methane was produced, and the result is presented in figure 1.

The subjects of experimentation were steers and cows of beef and of dairy breeds, and the rations fed were widely diverse as to intimate composition, but all were composed of normal, cattle feeding stuffs, and nearly all were mixed rations of concentrates and roughages.

The relationship thus demonstrated is not a direct proportion, but is expressed by the formula, $E = 4.012\,X + 17.68$, in which E signifies grams of methane produced, and X represents hundreds of grams of carbohydrate digested, the regression coefficient being computed by the method of least squares.

This formula was derived from 130 measurements of methane, by combustion, each representing 2 or 3 days of continuous collection, the intake of digestible carbohydrate being 900 to 5800 gm. per day. The formula, therefore, applies only to quantities of digestible carbohydrates within these limits.

The average difference between the observed and the computed values in this series of 130 determinations was 8.4%, and the coefficient of correlation between carbohydrate digested and methane produced was 0.94.

While methane production has never been computed in the research work of this laboratory, and the computation of quantities of methane produced, for critical purposes, is not advocated, this formula is proposed as a means of computing methane for uses which may be served by approximate values.

INDEX

SUPPLEMENT

PROCEEDINGS OF THE SEVENTH ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

The seventh annual meeting of the American Institute of Nutrition was held in New Orleans at the Jung Hotel on March 13, 1940. One hundred and ten members and 340 guests registered.

COUNCIL MEETING

The Council meeting was held at the Jung Hotel, Tuesday evening, March 12, at 6:30 P.M. All members were present. Formal actions of the Council are reported in the minutes of the Business Session.

SCIENTIFIC SESSIONS

The scientific program began at 9:00 A.M. and proceeded on schedule time. All the papers listed on the program were given. President Sherman presided at the morning session and Vice-President Carpenter at the afternoon session. The Symposium program held in the evening under the chairmanship of President Sherman was attended by about 800 persons and lasted for approximately 1½ hours. The carefully prepared papers provided an integrated program on the problem of better health which proved unusually interesting and thought-provoking.

BUSINESS SESSION

The business meeting was called to order by President Sherman at 11:30 A.M., March 13th. The reading of the minutes of the previous annual meeting was dispensed with since they had been published in the Journal.

1

The Secretary announced that, in accordance with the by-law providing that members in good standing who have reached the age of 65 years shall become emeritus members, A. J. Carlson, E. B. Hart, J. R. Murlin, and Mary S. Rose have attained this rank of service. The following resignations were reported, effective June 30, 1940: Oscar M. Schloss and C. A. Lilly.

The Secretary announced that the results of the mail vote relative to the question of making application for membership in the Federation were as follows: 158 affirmative and 7 negative; that the application had been made accordingly and would presumably be acted upon at the current meeting of the Federation. He stated that the correspondence dealing with this matter, most of which had been circulated to the membership, had been filed in the minutes of the Council. Upon recommendation of the Council it was voted that, in view of this pending application to the Federation, the questions of time, place and nature of the program for the 1941 meeting be left to the Council with power.

The Secretary announced that the Committee of Judges for the 1940 Mead Johnson Award consisted of F. F. Tisdall, chairman, E. V. McCollum, W. C. Rose, H. T. Parsons, and H. B. Lewis, and that its report which had been approved by the Council would be made at the Dinner.

By request of the Council the Secretary read excerpts of a letter from Dr. Icie Macy Hoobler relative to the recent "White House Conference on Children in a Democracy." The letter cited the recommendation of this conference that "in recognition of the fundamental importance of nutrition to the health of children, the President appoint a National Nutrition Committee, composed of scientists, physicians, agricultural experts, consumers' representatives, teachers, and administrators." The letter also expressed the conviction that the Institute should get behind this great movement which should give impetus to a national program of action and which would mean more food of the right kind for more children.

The Secretary announced that in the absence of any specific provision in the By-Laws the Council had established the fiscal year of the Institute as March 1–February 28.

The report of the Treasurer was read by W. H. Sebrell. The auditors, T. M. Carpenter and P. E. Howe, previously appointed by the President, reported that they had examined the Treasurer's books and found them correct. It was moved and carried that the report of the Treasurer be accepted.

G. R. Cowgill reported as Editor. The items mentioned by him are included in the formal report of the Editorial Board, which is included later.

The Secretary reported that during the past year Dr. Cowgill, upon being elected Editor of the Journal, had resigned as Treasurer and that, in accordance with the By-Laws, the nominating committee had selected W. H. Sebrell to fill the vacancy created by Dr. Cowgill's resignation. The Secretary also announced that the National Savings and Trust Company of Washington, D.C. had been designated by the Council as the official depository for the funds of the Institute.

The Tellers, R. C. Lewis and W. D. Salmon, reported that in the annual election 96 votes had been cast with the following choices:

> President—T. M. Carpenter
> Vice-President—A. G. Hogan
> Treasurer—W. H. Sebrell
> Secretary—L. A. Maynard
> Councillor—Lydia J. Roberts
> Associate Editors (for 4 years)—Lela E. Booher
>   H. B. Lewis
>   A. T. Shohl
> Associate Editor (for 1 year)—E. B. Forbes

Dr. E. M. Nelson made the following recommendation in behalf of the Committee on Vitamin Nomenclature:

> "It is recommended that the name PYRIDOXINE be adopted for the compound which has been identified as 2 methyl 3 hydroxy 4,5 di-(hydroxymethyl) pyridine and which has previously been designated by several names including vitamin B_6."

It was moved and carried that this recommendation be adopted. Dr. Nelson also reported that a name for vitamin K was under consideration.

Upon recommendation by the Council it was voted to ask the President to appoint a Committee to consider desirable changes in the Constitution and By-Laws. President Sherman appointed the following as members of this Committee: A. H. Smith, Chairman, Grace MacLeod, and L. A. Maynard.

President Sherman announced the appointment of the following Nominating Committee for 1940–41: H. H. Mitchell, Chairman, P. M. Nelson, Jennie Rowntree, C. E. Bills, G. O. Burr.

The Council recommended that the following candidates be elected to membership:

E. W. Crampton	Robert C. Lee
L. A. Crandall	L. L. Madsen
H. G. Day	Lane A. Moore
Lillian N. Ellis	E. S. Nasset
Margaret L. Fincke	P. V. Pearson
H. H. Gordon	Sybil L. Smith
A. Baird Hastings	

It was moved and seconded that the Secretary cast a unanimous vote for the election to membership of the candidates approved by the Council. The motion carried and the Secretary declared the candidates duly elected members of the American Institute of Nutrition.

It was voted to refer to the new Committee on Revision of the Constitution and By-Laws the question of providing a choice in the nominations made for at least one of the elective offices.

It was moved, seconded, and unanimously voted that the Secretary be instructed to write a letter to J. R. Murlin expressing the deep appreciation of the Institute for his pioneer work in founding The Journal of Nutrition and for his long, devoted and outstanding service as Editor.

It was moved and carried that the Secretary be instructed to extend for the members of the American Institute of Nutrition and their guests their sincere thanks to the Local Committee and the management of the Jung Hotel for the excellent arrangements provided for the meeting and for the many courtesies extended.

The meeting adjourned at 12:30 P.M.

LUNCHEON

The noon luncheon in the Mirror Room was attended by 132 members and guests.

DINNER

The subscription dinner held in the Jung Roof was attended by 231 members and guests.

The presentation of the Mead Johnson and Company Award for Research on the Vitamin B Complex for 1940 was made, in behalf of the Institute, by E. V. McCollum, a member of the Committee of Judges. One-half of the Award was presented to W. H. Sebrell for his work on riboflavin deficiency in man. In response, Dr. Sebrell paid tribute to the pioneer work of Dr. Goldberger and spoke feelingly of the inspiration and guidance he had received from this outstanding investigator. He also acknowledged the devoted assistance of his associate, R. E. Butler.

The other half of the Award was divided among the following investigators for their work on the isolation, structure, and synthesis of vitamin B_6: J. C. Keresztesy, J. R. Stevens, S. A. Harris, E. T. Stiller, and Karl Folkers. Dr. Keresztesy responded in behalf of this group. He related the thrills experienced as the various discoveries were made. He emphasized that they were the result of close team work and he paid tribute to the cooperation of both the officers and other employees of Merck and Company.

EDITORIAL BOARD

Dr. John R. Murlin resigned as Editor at the last annual meeting. The Editorial Board elected the new editor in June, 1939. The Wistar Institute asked Dr. Murlin to see through the press papers in the issues of The Journal of Nutrition up to and including that of October, 1939, which was done.

During the period of 5 months (November, 1939 to March, 1940) under the new editorial management forty-seven articles were published. Twenty-three papers were rejected for various reasons and one was withdrawn by its author. For this 5-month period the average number of pages per article proves

to be 10.8, indicating that the policy of the Editorial Board of making this average approximate the figure 10 is being followed. The number of articles per volume continues to increase slightly. In the last annual report (see Supplement, J. Nutrition, vol. 17, June, 1939) data are tabulated covering volumes 13 to 17, inclusive and showing that the number of articles per volume has risen from 48 to 55; volume 18 carried 56 papers. Two articles from foreign countries were accepted.

Beginning with volume 19, the first issue of which appeared in January, 1940, a new picture, that of Claude Bernard, has appeared on the cover page. This change was in keeping with the policy of using a new picture every 3 years or every six volumes. The January, 1940 issue also carried another change with respect to the kind of paper used in printing the Journal. The type of stock now used is tougher and has certain other advantages over the paper formerly used.

Hitherto The Journal of Nutrition has not been copyrighted. The publisher, The Wistar Institute, recently notified the editor that, beginning with the July, 1940 issue, all issues will be copyrighted. This will serve to protect both authors and publishers against any unauthorized reprinting of articles that have appeared in the Journal.

Discussion with the publisher of the question of printing three volumes a year, or enlarging the present two volumes has continued, but no change has been decided upon as yet. Until greater financial support can be secured for the Journal, it will be difficult to make such a change without appreciably increasing the cost to subscribers.

During the luncheon hour on March 13, 1940, a meeting of the Editorial Board was held at the Jung Hotel. The problems facing the editor in making decisions on papers, the ways in which members of the Board can assist in their solution, and related topics were discussed.

Respectfully submitted,

L. A. MAYNARD
American Institute of Nutrition

ABSTRACTS OF PAPERS

Pantothenic acid and its nutritional significance. Roger J. Williams (introduced by Jet C. Winters), Department of Chemistry, University of Texas, Austin.

The discovery and isolation of the vitamin which we have named pantothenic acid came about unconventionally inasmuch as it resulted from experimentation with microorganisms rather than with animals.

While a number of the recognized vitamins (thiamin, riboflavin, nicotinamide, adermin) have profound physiological effects on microorganisms, pantothenic acid is the only one discovered and isolated using microorganisms for biological testing.

The general importance of pantothenic acid in the whole field of biology is indicated by its presence in all tissues, and in every form of plant, animal and bacterial life examined. It likewise affects the respiration of widely different tissues. Its importance in the nutrition of the chick has been fully demonstrated, and its indispensability for rats seems certain. Its presence in the food is evidently unusually important during the early stages of growth of mammals. Milk is relatively rich. It is difficult to deplete rats, after weaning, with respect to this vitamin. They obtain it from milk and store it in the liver and other tissues, where it is built into colloidal constituents. Chicks, however, are easily depleted and the content of their tissues may be diminished 50% or more during a 14-day feeding period. Squabs after a period of growth on ''crop milk'' can live for an extended time receiving only small amounts of pantothenic acid.

The chemical structure of pantothenic acid will be elucidated.

Experimental vitamin B_1 deficiency in man. R. D. Williams (by invitation), H. L. Mason (by invitation), and R. M. Wilder, Mayo Foundation, Rochester.

A diet containing less than 0.1 mg. (30 I.U.) vitamin B_1 (thiamin) daily but adequate in other respects was consumed by eight active young women. The purpose was to produce and study a state of vitamin B_1 deficiency unassociated with disease states, starvation or unusual environmental stress. Two subjects were maintained on an identical regime with the exception that a maintenance dose of thiamin was given by mouth.

Urinary excretion of thiamin was determined at weekly or daily intervals and related to the appearance of fatigue, anorexia, achlorhydria, electrocardiographic changes, disturbances of carbohydrate utilization as reflected in diabetic type blood sugar time curves and abnormal accumulation of metabolites, pyruvic and lactic acids. Fatigue was correlated with record of work and studies of oxygen deficit. The time of appearance of symptoms and signs of the deficient state varied widely, but in every case urinary excretions of thiamin fell to extraordinarily low levels. Striking electrocardiographic changes and other manifestations incident to the induced state of deficiency quickly disappeared when relatively small doses of thiamin were given by injection. The observations encourage us to believe that estimates of thiamin excretion before and after a standard dose of thiamin may serve as an index of borderline states of vitamin B_1 deficiency.

7

Metabolism of citric acid and of food citrate in infants. A. H. Smith, D. J. Barnes (by invitation), and C. E. Meyer (by invitation), Department of Physiological Chemistry, Wayne University College of Medicine and Harper Hospital, Detroit.

Eight infants varying from 3½ months to 12 months of age were maintained on an adequate diet of cereal, milk, B-lactose and strained fruits. Quantitative collections of urine and feces were made separately in 3-day periods. On the diet alone the excretion of citrate in the urine varied from 12.9 to 6.6% of that ingested and in the feces, 13.0 to 1.9%. During the experimental period 365 mg. of citric acid per kilogram body weight was given by gavage, in addition to the basal diet. Absorption of this increased amount of citrate varied from 76 to 99% of the total consumed; the fecal output was 24.1 to 0.8% (diarrhea being correlated with the high fecal loss) and urinary excretion varied from 3.8 to 0.9%, of the total intake. The data show that foods ordinarily given to infants contain considerable citrate and that this citrate largely disappears in the course of metabolism. Addition of considerable amounts of free citric acid to the basal diet results in a greatly diminished excretion of citrate in the urine and .(if diarrhea is absent) in the feces. The data can be interpreted to indicate that free citric acid is not only completely metabolized by the infant but that it causes a reduction in the amount synthesized in the course of metabolism.

The biological activity of pyrazine acids and quinolinic acid. W. J. Dann, H. I. Kohn (by invitation), and P. Handler (by invitation), Department of Physiology, Duke University, Durham.

Under our standard conditions a dog with acute blacktongue can be cured with 2 mg. nicotinic acid per kilogram body weight daily for 5 days. This amount of nicotinic acid could not be effectively replaced by pyrazine monocarboxylic acid up to 10 mg., by pyrazine 2,3-dicarboxylic acid up to 14 mg. or by quinolinic acid up to 14 mg. With each compound the results were irregular; some dogs showed remission of mouth symptoms but did not regain normal weight, while others died.

In preventive tests it is known that 0.13 mg. of nicotinic acid per kilogram daily will maintain a dog free from blacktongue over several months (Birch; J. Nutrition, vol. 17, p. 281). We have found that 3 mg. pyrazine monocarboxylic acid, or 4 mg. pyrazine 2,3-dicarboxylic acid, or 2 mg. quinolinic acid per kilogram daily did not prevent the onset of blacktongue within 2 months.

Nicotinic acid in large doses by mouth causes an increase in the V-factor (coenzymes I & II possibly plus unknown related substances) of human blood cells in vivo; when defibrinated human blood is incubated with nicotinic acid in vitro the cells synthesize V-factor (Kohn and Klein; J. Biol. Chem., vol. 130, p. 1). Neither of these effects followed when nicotinic acid was replaced by pyrazine monocarboxylic acid, by pyrazine 2,3-dicarboxylic acid or by quinolinic acid in equivalent or greater amount.

Man's utilization of dietary calcium. J. P. Outhouse, R. Mills (by invitation), H. Breiter (by invitation), B. McKey (by invitation), E. Rutherford, W. Armstrong (by invitation), and H. H. Mitchell, Departments of Home Economics and Animal Husbandry, University of Illinois, Urbana.

In these studies calcium balances have been determined at two levels of intake, both of which were lower than that required for maximum retention by the child,

or for equilibrium by the adult. Percentage utilization values were computed on the basis of the differences in calcium retention (or in losses) on the two levels of intake. The length of the periods ranged from 35 to 70 days each. Seven little boys, given $CaHPO_4$, in addition to their basal food, were able to utilize only 19.0% of its calcium. Five of these children, while receiving $CaHPO_4$, were given a daily supplement of lactose (C.P.) in an amount equivalent to that in 750 cc. milk. In all five subjects the calcium retentions were increased, the increase ranging from 8 to 83%; the average value for the group was 33%.

The utilization by seven adults of the calcium of carrots has been studied. Seven hundred grams carrots were eaten daily; the range in calcium utilization values was from 0 to 33% with an average value of 14.0%. The low values for six of the subjects necessitated a determination of the extent to which they utilized the calcium of milk. Preliminary data indicate a higher utilization of milk calcium.

Human nutritional anemia in Florida. O. D. Abbott (introduced by L. A. Maynard), C. F. Ahmann (by invitation), and M. R. Overstreet (by invitation), Division of Nutrition, Florida Agricultural Experiment Station, Gainesville.

An examination of 2277 rural school children in Florida showed that 52% of them were anemic, 25% had borderline anemia, while only 22% had normal hemoglobin values. It was found that in those districts, where the predominant soils were classed as deficient in regard to salt lick of cattle, from 52 to 96% of the children were anemic but in districts where the soils were classed as protected, from 0 to 23% were anemic. The iron content of turnip greens (selected as an index food) varied from 258 p.p.m. when grown on protected soils to 56 p.p.m. when grown on deficient ones. The iron content of the diets of children obtaining the greater part of their food from these soils varied likewise. That the anemic condition was due to a mineral deficiency was demonstrated by treating 400 anemic children with ferric ammonium citrate. Within 4 to 6 weeks after beginning treatment all the subjects showed marked improvement. It appears that soil deficiencies operating through plants grown thereon and ultimately on the health of the people is a factor that should be considered in any section where nutritional anemia is endemic.

The relation of dietary protein to hemoglobin formation in the rat. A. U. Orten (by invitation) and J. M. Orten, Department of Physiological Chemistry, Wayne University College of Medicine, Detroit.

The relation of dietary protein to hemoglobin formation in the rat is being investigated. Animals at weaning (21 days) were fed a synthetic diet adequate except for a low level of protein of excellent quality (lactalbumin). Special attention was given to assure the daily consumption of an abundance of all the known vitamin factors.

At 100 days of age, the "low-protein" animals appeared in good condition, although increase in body weight had been almost entirely inhibited. Blood studies showed, however, the existence of a typical hypochromic anemia, characterized by a nearly normal red cell count, a low hemoglobin value, and an increased proportion of reticulocytes. Calorie control rats receiving exactly the same quantities of all of the dietary constituents except the amount of protein (increased at the expense

of carbohydrate) showed a normal blood picture. Furthermore, the feeding of an increased amount of protein (again at the expense of carbohydrate) with no increase in calories or inorganic matter, promptly corrected the anemia. Increasing the iron intake, on the other hand, had no effect. Thus, the anemia is clearly referable to the low protein intake.

Additional experiments designed to determine the possible effect of the administration of individual amino acids, including glycine, leucine, tryptophane, histidine, cystine, methionine, valine, glutamic acid, proline, phenyl alanine, tyrosine, and lysine, will be discussed.

Carbohydrate combustion in man after oral versus intravenous administration of dextrose. Thorne M. Carpenter and Howard F. Root, Nutrition Laboratory of the Carnegie Institution of Washington and Baker Clinic of the New England Deaconess Hospital, Boston.

In observations on certain diabetic subjects, there was no definite rise in respiratory quotient after intravenous injection of dextrose. As a control, comparison was made of the effects of oral and intravenous administration of dextrose with four normal men. The respiratory exchange was determined in three 10-minute periods with the men sitting, post-absorptive, and in nine 15-minute periods after dextrose. In one series of experiments, 50 gm. of dextrose in 350 cc. of water was taken orally. In another, 50 gm. of dextrose in 500 cc. of a normal saline solution was injected intravenously. After sugar was given, the respiratory quotient showed a definite rise and nearly the same course in both series. The oxygen consumption also increased in both series. The increases above the base lines in the calculated carbohydrate combustion ranged from 7.1 to 15.0 gm. during 2¼ hours after oral ingestion and from 5.5 to 15.7 gm. after intravenous administration. The increases after oral ingestion averaged 1.6 gm. higher than those after intravenous introduction. The smallest difference was found with the subject showing the greatest increases above the base line. The increases in heat production ranged from 8.0 to 27.5 cals. after oral ingestion and from 6.8 to 18.4 cals. after intravenous injection. The average difference between the two methods was 5.0 cals. After intravenous injection, all subjects eliminated sugar in urine.

Vitamin K. E. A. Doisy (introduced by H. S. Mitchell), S. B. Binkley (by invitation), D. W. MacCorquodale (by invitation), R. W. McKee (by invitation), and S. A. Thayer (by invitation), Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis.

Following the development of a quantitative procedure for the assay of vitamin K, the natural antihemorrhagic factors have been isolated from alfalfa and from putrefied sardine meal by a process in which the important feature was chromatographic adsorption on decalso and permutit. The former was called vitamin K_1 ($C_{31}H_{46}O_2$), and the latter, vitamin K_2 ($C_{41}H_{56}O_2$).

The oxidation of vitamin K_1 gave 2-methyl-1,4-naphthoquinone-3-acetic acid and a ketone which proved to be identical with the ketone from phytol. Synthesis of 2-methyl-3-phytyl-1,4-naphthoquinone gave a product which was identical with the natural vitamin K_1.

Degradation has given conclusive evidence that vitamin K_2 is also a 2,3 disubstituted 1,4-naphthoquinone. Ozonolysis of diacetyl dihydrovitamin K_2 with decom-

position of the ozonide with zinc has given the same ring compound, 1,4-diacetoxy-2-methyl-naphthalene-3-acetaldehyde, that was obtained from vitamin K_1. This clearly indicates that vitamin K_2 has the active 2-methyl-1,4-naphthoquinone structure and that it differs from vitamin K_1 only in the substituent in the 3 position.

Following our discovery of the nature of the ring structure of the natural vitamins, it was shown that several simple naphthoquinones possess antihemorrhagic properties. Moreover, two compounds which are closely related to the very active 2-methyl-1,4-naphthoquinone, i.e., 1,4-dihydroxy-2-methylnaphthalene and 4-amino-2-methyl-1-naphthol hydrochloride are approximately as potent. Due to their solubility in water these compounds are important for intravenous therapy.

Comparative activities of certain antihemorrhagic quinones. H. J. Almquist and A. A. Klose (by invitation), Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley.

The authors have assayed by quantitative methods various quinones with antihemorrhagic activity in an effort to establish the comparative potencies of these compounds according to a common reference standard and assay methods. The methods involve no master curves or arbitrarily chosen values. Data have been obtained on vitamin K_1 (natural and synthetic), vitamin K_2 and other compounds that have been mentioned in the literature. 3-nitro-, 3-amino, 3-palmityl-, and 8-sulfonic acid derivatives of 2-methyl-1,4-naphthoquinone have been synthesized.

A comparison of similar compounds suggests that potency decreases as relative water solubility increases. 1,4 reductive acetylation decreases activity to from one-half to one-third of the original value. The activities of vitamins K_1 and K_2 are in direct proportion to their contents of the naphthoquinone nucleus. The long side chains of these vitamins are non-essential for potency and are probably split off in the metabolism of the vitamin. The antihemorrhagic potency of compounds appears to depend upon the presence of, or ready conversion to, the active configuration as present along the 1,2,3,4 positions of 2-methyl-1,4-naphthoquinone.

The potencies of vitamins K_1 and K_2 were found to be, respectively, 290 and 230 methyl naphthoquinone units per milligram.

Biological synthesis of ascorbic acid. H. E. Longenecker (by invitation) and C. G. King, Chemistry Department, University of Pittsburgh, Pittsburgh.

The occurrence of ascorbic acid in nearly all living cells, irrespective of nutrient requirement, provides an indication that the acid can be synthesized from other metabolites by a great many plants and animals. A number of compounds that stimulate synthesis and excretion in experimental animals have been identified. Comparative tests with different animals show wide variations in response to different agents. Some of the most active compounds are in common pharmacological and clinical use for other purposes, especially as antipyretics, analgesics and hypnotics. The response to specific stimulants can be very markedly influenced —essentially controlled—by the diets of experimental animals such as the rat.

Further studies on the relationship of choline and betaine in the utilization of homocystine. Vincent du Vigneaud, J. P. Chandler (by invitation), and A. W. Moyer (by invitation), Cornell University Medical College, New York.

In a previous investigation evidence was obtained that the presence of choline or betaine in the diet was necessary to enable homocystine to replace methionine in

the diet. It was therefore suggested that choline brought about the methylation of homocysteine to methionine and that the reaction might be reversible with methionine acting as a precursor of choline in the body in the sense of supplying necessary methyl groups. The present report deals with a closer examination of the relationship of choline and related substances to the utilization of homocystine.

Experiments have been carried out to ascertain whether choline would be effective by injection. The amounts required were relatively of the same order of magnitude as by mouth, indicating that the formation of methionine was not due to the action of intestinal bacteria.

A careful comparison of the effectiveness of choline and betaine was also made. By either route betaine showed a delayed and somewhat less efficient action than choline. This was particularly true of very young animals of 21 to 25 days of age.

An extended study of the relationship of structure of choline to the reaction in question will be presented. Studies are also under way in an attempt to show by direct proof the conversion of homocystine to methionine in the presence of choline in vivo and of the significance of the methyl group of methionine for the synthesis of choline.

Observations on the relation of vitamin P to scurvy. E. W. McHenry and H. M. Perry (by invitation), School of Hygiene, University of Toronto, Toronto.

The principal physiological effect of vitamin P was described by Szent-Györgyi as the maintenance of resistance of capillary walls and the consequent prevention of hemorrhage. Several workers, notably Zilva, have failed to substantiate the claims for vitamin P but Scarborough has recently provided evidence for the existence of such a factor. Using guinea pigs, in which hemorrhage is a marked feature of scurvy, we have studied the action of purified preparations of vitamin P. These have had no effect in preventing increased capillary fragility, which is produced by a lack of ascorbic acid and promptly cured by that substance. Paired feeding experiments show that inanition is not a factor. In an attempt to verify these observations in humans we have employed a simple, standardized test for capillary fragility. In fifty-eight normal persons the results over an extended period have been so variable that no significance could be attached to them. This is in agreement with the work of Weld. It is concluded that deficiency of vitamin P is not a factor in producing hemorrhages in scorbutic guinea pigs, that a deficiency of ascorbic acid is alone responsible, and that a capillary fragility test is not a reliable indication of ascorbic acid deficiency in humans.

Unavailable carbohydrate balances of children. F. C. Hummel (by invitation), M. L. Shepherd (by invitation), and I. G. Macy, Research Laboratory, Children's Fund of Michigan, Detroit.

Unavailable carbohydrate balances for ten normal children, four girls and six boys, ages 5 to 8 years demonstrate the variations in intake, physiological response and carbohydrate disappearance in the alimentary canal which occur consequent to a uniform daily diet composed of sufficient nutrients to meet the individual's requirements of growth, activity and well being.

During the observation of 220 consecutive days (forty-four 5-day balances) for each child the average total fiber consumed by the subjects per day varied from

5.55 to 6.49 gm. of which 2.08 (SD = 0.07) to 2.42 (SD = 0.26) gm. were cellulose and 2.00 (SD = 0.19) to 2.51 (SD = 0.16) gm. hemicellulose. The consequent laxation rate varied from 1.1 (SD = 0.09) to 2.5 (SD = 0.70) and the disappearance of cellulose from 1.21 (SD = 0.42) to 1.95 (SD = 0.2) gm. and of hemicellulose 1.09 (SD = 0.33) to 1.54 (SD = 0.27) gm. Individual differences exist among children in their ability to break down cellulose and hemicellulose in their respective digestive tracts. In some cases as much as 80% of cellulose is destroyed in its passage through the tract in contrast to low values of 53% while hemicellulose is somewhat lower and less variable in its decomposition (49 to 61%).

The above results will be considered in relation to the individual's gastrointestinal motility, daily fecal excretion (wet weight, total solids, nitrogen and fat content) and other pertinent physiological findings.

The rate of digestion of some natural and synthetic fats in the rat. H. J. Deuel, Jr., and L. F. Hallman (by invitation), Department of Biochemistry, University of Southern California Medical School, Los Angeles.

Certain natural fats were fed to fasting rats in amounts of 1 cc. and the amount of undigested fat remaining in the gut was determined at 3, 4½, and 6 hours thereafter by a procedure described earlier.[1] No differences were noted in the rate of digestion of a hydrogenated cottonseed fat, butter, coconut oil or wintered cottonseed oil. Rape seed oil on the other hand was absorbed more slowly. Hydrogenated cottonseed fat and rape seed oil were absorbed more rapidly when fed in higher doses.

In the experiments with synthetic fats, it was found that best recoveries were obtained when the gut was flushed with diethyl ether alone. The fats were administered in doses of 300 mg. per 100 sq.cm. and the animals killed after 3 hours. The fats having even-chain fatty acids (triacetin, tributyrin, tricaproin, and tricaprylin) were absorbed at about the rate of natural fats (60–50 mg. per 100 sq.cm. per hour) while the fats having an odd number of carbon atoms (tripropionin, trivalerin, and triheptylin) disappeared at approximately one-half the rate of the even-chain fats.

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[1] Deuel, H. J., Jr., Hallman, L. F., and Quon, S., J. Biol. Chem., vol. 128, XIX (1939).

Muscular dystrophy in rabbits and the autoxidation of animal fats. H. A. Mattill, Biochemical Laboratory, State University of Iowa, Iowa City.

On a synthetic diet containing lard and cod liver oil and otherwise adequate except for the absence of vitamin E, growth ceases in young rabbits (700–900 gm.) after 10 to 20 days, and dystrophy soon appears. Such diets supplemented by 3–5% of wheat germ oil usually allow normal growth and the appearance of dystrophy is postponed. On standing the vitamin E content of such diets gradually declines. This loss of vitamin E may be delayed by the use of appropriate stabilizers against oxidative rancidity.

The water-soluble factor is apparently supplied in adequate amounts by 5% of yeast in the diet. Wilson's defatted liver powder contains no vitamin E. The water-soluble factor in yeast is much less vulnerable than vitamin E when in contact with autoxidizing fats. α-Tocopherol acetate[1] is much less effective when given parenterally or subcutaneously than when given by mouth.

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[1] Hoffmann-La Roche.

Relation of perosis to unrecognized vitamins. A. G. Hogan, L. R. Richardson (by invitation), and Homer Patrick (by invitation), Department of Agricultural Chemistry, University of Missouri, Columbia.

It is now well established that the perosis which develops in chicks on certain rations can be prevented by including additional manganese in the ration. During the course of our efforts to develop adequate simplified rations for the chick, perosis was occasionally encountered even though the diets contained much more manganese than is commonly recommended as a preventative. This observation suggested that an organic factor, as well as manganese, is concerned in the syndrome and an attempt was made to prepare vitamin carriers that do not contain it. Dried liver was extracted first with hot alcohol and then with hot water. The alcohol extract contains all of the substances that prevent perosis. The water extract and the liver residue contain little or none, and they are included in the perosis producing ration. Thiamin, riboflavin, and vitamins A and D are also included, and the ration is improved by adding B_6. Eighty-eight chicks, from ten different hatches, have received this ration and 100% developed marked cases of perosis. Two hundred and forty-eight chicks have received a similar ration, except that it contained the alcohol extract, and none developed perosis. It is concluded that the chick requires a previously unrecognized vitamin, provisionally designated as B_p.

The effect of nicotinic acid and coramine on experimental blacktongue and human pellagra. D. T. Smith (by invitation), J. M. Ruffin (by invitation), and S. G. Smith, Department of Medicine, Duke University School of Medicine, Durham.

A study of over 400 cases of pellagra indicates that the cardinal symptoms are anorexia, dermatitis, glossitis, diarrhea and dementia. Pellagrins may have multiple secondary deficiencies especially among members of the B complex, e.g. B_1, riboflavin and B_6. The administration of nicotinic acid or coramine cures the primary symptoms, and usually the secondary ones as well, probably by stimulating the ingestion and assimilation of more food. However, severe secondary deficiencies must be corrected before the patient will respond properly to nicotinic acid.

In dogs with experimental blacktongue the daily minimal curative dose of nicotinic acid is 0.5 mg. per kilogram. To obtain the same effects with coramine 7.25 mg. per kilogram is required. Multiples of the minimal dose of both drugs give no more rapid improvement and are not stored by the body.

When dogs with experimental blacktongue develop secondary deficiencies of B_1 and riboflavin they fail to gain weight with nicotinic acid therapy until these deficiencies are corrected.

Solutions of nicotinic acid may be sterilized by boiling or autoclaving without loss of potency and may be administered orally, intramuscularly, or intravenously. Doses three times the minimal dog dose (1.5 mg. per kilogram) or approximately 100 mg. per day produce prompt and dramatic cures in the most severe cases of pellagra. Twice the minimal dog dose of coramine or approximately 750 mg. per day has also given entirely satisfactory results.

ABSTRACTS OF PAPERS READ BY TITLE

Spectroscopic studies of fatty acids. R. H. Barnes (by invitation), J. P. Kass (by invitation), E. S. Miller (by invitation), and G. O. Burr, Departments of Physiology and Botany, University of Minnesota, St. Paul.

Recently physiological studies have been aided by the use of tagged or labelled molecules. In lipid metabolism radioactive phosphorus has been used. Iodized fatty acids, highly unsaturated acids, elaidic acid, acids containing deuterium, and spectroscopically active acids will label both phospholipids and neutral fats. Spectroscopically active acids have proved especially sensitive indicators because of the high precision of the spectroscope and the small amounts required.

Normal tissue fats have very low absorptive values at 2300 A.U. (lard = about 3). Corn oil can be activated to a value of 400, which is so high that a trace of it can be detected in the normal fat. The activated fatty acids are usually administered as methyl or ethyl esters. They are not at all toxic and are apparently normally metabolized.

Recent studies have given very regular curves for rates of absorption and transport to the liver. In the intestinal mucosa the neutral fats show maximum spectral absorption within ½ hour, while the phospholipids give a smooth rising curve which is approaching a limit of tagged acid interchange only after 14 hours. This and other experimental data are shown and discussed.

The method of activation of fatty acids has been made quantitative so that it is possible to analyze an oil accurately by this procedure. The method of analysis is given in detail.

A deficiency disease in chicks prevented by dl-alpha tocopherol. H. R. Bird (by invitation), and T. G. Culton (by invitation), (introduced by O. L. Kline), Department of Poultry Husbandry, University of Maryland, College Park.

Chicks were fed from hatching time a diet of dried skim milk 54%, dextrinized starch 44%, ground limestone 1% and NaCl 1% with the following additions of mineral salts: ferric citrate 0.12%, $CuSO_4 \cdot 5 H_2O$ 0.0012% and $MnSO_4 \cdot 4 H_2O$ 0.012%. Cod liver oil was administered by pipette thrice weekly. When certain samples of dried skim milk were fed as a part of this diet there was a high incidence of generalized edema in chicks 3 to 8 weeks of age, followed by death in all cases. The most consistent post mortem finding was a very great distension of heart and pericardium, the latter being filled with exudate. In most cases there was extreme ascites and subcutaneous edema, and frequently edema of the brain and lungs, extreme coronary and intestinal hyperemia, and deposition of urates in ureters and kidneys.

This condition was effectively prevented, first, by inclusion of 3% of dehydrated grass in the diet, and later by the administration in cod liver oil of synthetic dl-alpha tocopherol at a dosage approximating 7.5 micrograms per gram live weight per day. The tocopherol was kindly furnished by Merck and Co. and its use was suggested by Dam's[1] report of its effectiveness in preventing the "alimentary exudative diathesis" which he observed in young chicks.

[1] Dam, H., Nature, vol. 143, p. 810 (1939).

The effects of alimentary and adrenalin hyperglycemia on total oxidation of carbohydrate in normal humans. J. W. Conn (by invitation), E. S. Conn (by invitation), and M. W. Johnston, Nutrition Laboratory, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor.

For 6 to 8 weeks, three normal young men were fed constant maintenance diets. Activity from day to day was constant. Twenty-one 4-hour experimental periods of continuous indirect calorimetry by the open-circuit method were done under conditions of fasting, glucose orally, adrenalin subcutaneously and the latter two simultaneously. Under the same conditions blood sugar curves were obtained.

Results and conclusions

The blood sugar level does not control total oxidation of glucose in normal humans. After adrenalin with levels reaching 200 mg. %, less glucose was oxidized than in the fasting state at levels of 80 mg. %. Levels of 350 mg. % produced by adrenalin plus glucose resulted in no greater oxidation of glucose than levels of 170 mg. % produced by glucose alone. With the same degree of hyperglycemia, over twice as much glucose was oxidized when the hyperglycemia was produced by ingested glucose as when produced by adrenalin.

Under all conditions studied, oxidation of glucose, when influenced by adrenalin, was decreased, despite the plethora of glucose in the circulating blood.

The conflicting results of others, using hepatectomy, are reconcilable if one assigns to the liver of the normal intact animal the function of initiating the mechanism which determines how much of the total heat produced will be derived from carbohydrate.

The liver, as the immediate receptor of the dietary or as influenced by abnormalities of carbohydrate metabolism, regulates carbohydrate oxidation in the animal body.

A study of vitamin C nutrition in a group of school children. Marian M. Crane (by invitation), P. W. Woods (by invitation), E. M. Waters (by invitation), and E. F. Murphy (by invitation), Children's Bureau, U. S. Department of Labor, Washington, and Maine State Bureau of Health and Maine Agricultural Experiment Station, Orono.

The state of nutrition with respect to vitamin C in a group of eighty-six rural Maine grade-school children was studied in the autumn (1938) and again in the spring (1939). Ascorbic acid in the blood plasma was determined by a modification of Ingalls' technique. Approximately 28% of the children had low values (less than 0.40 mg. per cent) in both autumn and spring, about 27% had intermediate values (0.40–0.79 mg. per cent) in autumn and low values in spring. Only two children had values consistently high enough to be considered evidence of adequate vitamin-C intake (0.80 mg. per cent or above) at both examinations.

Urinary tests of vitamin-C tolerance in forty-nine children generally supported the validity of plasma ascorbic acid determinations as evidence of the state of vitamin-C nutrition.

Examination of the eighty-six children by a dentist showed that approximately 29% had inflamed gums both in autumn and spring, and about 51% (an additional

22%) had inflamed gums at the spring examination. The association between mouth findings and plasma ascorbic acid values and the results of vitamin-C therapy administered to forty-one children with inflamed gums indicate that vitamin-C undernutrition is, in all probability, a factor in the causation of the inflammation.

The classical picture of scurvy was not observed in any of the children studied.

Study of the diets of a number of the children showed that probably not more than one child in seven was eating any food that is a good source of vitamin C as often as once a day.

Hemorrhagic adrenal necrosis in rats on deficient diets. F. S. Daft (by invitation), and W. H. Sebrell, National Institute of Health, Washington.

Rats on a diet deficient in the vitamin B complex were given supplements of nicotinic acid, choline, B_1, riboflavin and vitamin B_6 in various combinations. In many of the animals extensive hemorrhagic necrosis of the adrenal glands was found at death. When crystalline vitamin B_6 was used the lesions were seen earlier on a larger percentage of the animals. This increased incidence may be explained on the basis of the accentuation of the symptoms of one deficiency by the administration of another missing vitamin. The lesions were not seen when a crude liver or rice polishings extract was added in sufficient amounts. The chemical properties of the factor preventing the hemorrhagic adrenal necrosis and its identity or non-identity with known and postulated water soluble vitamins will be discussed.

Studies of the vitamin B-complex with chicks. T. H. Jukes, Division of Poultry Husbandry, University of California, Davis.

Experiments with a diet deficient in vitamin B_6 indicated that chicks require about 3 micrograms of this factor per gram of diet. The corresponding requirement for thiamin is about 1.5 micrograms; for riboflavin, 2.7 micrograms; and for pantothenic acid, 14 micrograms. An unknown water-soluble fraction is also needed. This was investigated with chicks on a simplified diet, supplemented with synthetic thiamin and riboflavin, and with rice bran filtrate to supply pantothenic acid. A fraction promoting growth and permitting survival was found present in corn and in extracts of yeast, alfalfa meal, liver and grass. The factor was largely destroyed by autoclaving the yeast. The factor was precipitated from aqueous solution by addition of alcohol or acetone. Attempts to fractionate yeast extracts resulted in large losses. Yeast fuller's earth eluates, potent sources of factor 2 for rats, produced very little response from the chicks. Additions of nicotinic acid or synthetic vitamin B_6 to the diet were ineffective. Addition of further amounts of thiamin improved growth and permitted survival; but when riboflavin and nicotinic acid were added in addition to thiamin, no improvement over the basal diet was noted.

The dietary requirement for the known B-factors is compared with the quantitative distribution of various members of the vitamin B-complex in certain foods, and the question of balancing a diet with respect to the individual B-factors is discussed.

The anti-cataractogenic action of certain nitrogenous factors. H. S. Mitchell, G. M. Cook (by invitation), and M. D. Henderson (by invitation), Nutrition Laboratory, Massachusetts State College, Amherst.

It was reported earlier that inadequate protein (5%) aggravated and that high-protein (45%) inhibited the development of cataract in rats on a 25% galactose ration. Having eliminated known vitamin entities as protective agents, the search has been for a specific nitrogenous factor.

Casein hydrolysate gave the same protection as an equivalent amount (15%) of commercial casein. Cystine and methionine gave only slight and inconsistent protection. Moreover when the sulfhydryl amino acids were made less available by feeding 100 mg. of iodoacetic acid per 10 gm. of casein, growth was retarded but cataract was not hastened. Urea at 3, 5 and 10% levels gave less protection than an equivalent amount of nitrogen fed as protein but more than the 5% casein basal ration. Choline was studied by feeding 1, 2, 4 and 8% in a 5% casein ration. Growth was progressively poorer the higher the level of choline fed and it exerted no protection against cataract.

When casein, deaminized by treatment with nitrous acid, furnished two-thirds of the 15% protein in the ration, growth was delayed but there was more protection against cataract than with commercial casein. On the other hand the prolonged exposure of certain proteins to dry heat appears to destroy the protective action in some more than in others and not in proportion to the growth-promoting power. Work on the identification of the protective factor is now in progress.

Anti-grey hair vitamin deficiency in dogs and silver foxes. A. F. Morgan, Department of Home Economics, University of California, Berkeley.

Eighteen young dogs from five litters and six young foxes of three litters were placed at 5 to 8 weeks of age on a purified diet of washed casein, cornstarch, sucrose, Crisco and salt mixture. They received separately daily additions of cod liver oil reinforced with carotene, crystalline thiamin chloride, riboflavin, vitamin B_6 concentrate or crystalline B_6. Some received in addition nicotinic acid and/or a yeast or rice bran filtrate concentrate.

Those which received all the supplements grew normally, matured sexually and retained their coat color. Those without either nicotinic acid or the filtrate factor grew fairly normally but after 3 to 4 months their hair showed definite and progressive depigmentation beginning at the follicles.

The animals which received nicotinic acid but no filtrate factor developed the depigmentation earlier and manifested bloody diarrhea, loss of appetite, cachexia and death within 3 months. If the missing factor was given, immediate restoration of appetite, growth and repigmentation of hair and skin occurred. One of the anti-grey factor-deficient foxes died suddenly after 6 weeks and the two remaining lost their hair but on administration of the missing factor grew a new coat of white instead of black fur.

When nicotinic acid only was withheld the dogs died after 3 to 6 months with inflamed mouths, bloody diarrhea and usually convulsions. Fading of the hair did not occur.

Apparently the two factors, nicotinic acid and the anti-grey filtrate factor are interrelated and to some extent antagonistic in function. Low urine volumes and

low blood chlorides were observed in the filtrate-factor deficient but not in the nicotinic acid deficient dogs.

Possible significance of fecal concentrations of the factor protective against egg white injury. H. T. Parsons, J. Gardner (by invitation), and C. T. Walliker (by invitation), Department of Home Economics, University of Wisconsin, Madison.

Feces produced on a series of raw egg white rations are non-potent when fed raw to test rats on raw egg white rations, but are highly potent fed in like manner after thorough, moist heating. Apparently mild hydrolysis in cooking the feces frees some protective factor held in non-absorbable form by some substance.

Feces produced on rations differing from the first series only in that the raw egg white has been replaced by either cooked egg white or raw egg white after the removal of a highly injurious fraction during dialysis, possess after cooking either no demonstrable potency or much less than that in feces produced on raw egg white rations. It is thought that fecal bacteria are not responsible for the difference in the effects in the two series inasmuch as the rations containing raw egg white are so closely similar. From these observations it is suggested as a tentative hypothesis that raw egg white derives its essential capacity to produce a pathological condition, not from its own possible lack of the protective factor in available form nor from the presence of a hypothetical antienzyme but from its characteristic in the digestive tract of combining with and holding in an unabsorbable form, the protective factor which originated either from the diet alone or, also, from an excretion into the digestive tract. The "curative" quality of cooked egg white probably depends on the destruction of such a capacity rather than on the formation of protective factor in the egg white itself by cooking.

Reproduction on "adequate" diets containing meat. P. P. Swanson and P. M. Nelson, Nutrition Laboratory of the Foods and Nutrition Department, Iowa State College, Ames.

In an experiment designed to study the nutritive value of dried autoclaved pork in a diet fortified with all known dietary essentials, extinction of the species occurs in the second generation when the rat is used as the experimental animal. Physiological disturbances causing the phenomenon are: acute and fatal pregnancy disease, either birth of non-viable young or failure of establishment of mammary function, and complete sterility in the second generation.

The correlation of the diet to the reproductive upset has been studied extensively. First, balance studies show that autoclaving does not reduce the biological value of the pork muscle. Second, peroxide values indicate that rancid fat is not the dietary offender. In this connection, assay proves that the ration is adequate in essentials possibly affected by rancidity (A and E). Third, the addition of B complex, liver extract, E, lactoflavin, K, choline, or lecithin reduces the efficiency of the ration. Fourth, the diet is markedly improved by the substitution of beef for pork in the formula or by supplementation with liver or a certain lipocaic preparation.

The liver addition prevents the degeneration of hepatic tissue associated with pregnancy disease, reduces the per cent of individual feti found resorbing at

autopsy prior to the birth of the second litter and permits normal birth and suckling of litters. The diet, therefore, is probably deficient in some unidentified factor or factors.

Availability of phosphorus in hays. D. E. Williams (by invitation), F. L. MacLeod, E. Morrell (by invitation), and H. R. Duncan (by invitation), Agricultural Experiment Station, University of Tennessee, Knoxville.

Evidence for difference in availability of phosphorus in low phosphorus hay versus high phosphorus hay of the same type was found with white rats. Hays fed were lespedeza sericea, alfalfa, soybean hay, and red clover. Nutritive quality of the diets and food consumptions of comparable animals were kept as uniform as possible. In the control diet, the phosphorus was supplied from a readily assimilable salt mixture in an amount approximately minimal for normal growth. In the hay diets, the same amount of phosphorus was supplied one-half by salt mixture and one-half by a large amount of low phosphorus hay in one case and by a smaller amount of high phosphorus hay in the other.

The animals on the low phosphorus hays were less thrifty than the controls or the animals fed the high phosphorus hay diets according to various criteria indicating rate of growth and phosphorus retention. Animals on the low phosphorus hay eliminated the largest amounts of feces.

Similar experiments, now under way, in which calves replaced rats apparently confirm the rat experiments. During 6 months' experimentation average gains in growth expressed by body weight in pounds, height and heart girth in inches, respectively, are for control calves, 139, 4.79, 5.71; for calves on low phosphorus red clover, 49, 1.71, 1.59; for calves on high phosphorus red clover 134, 3.87, 5.37.

ABSTRACTS OF SYMPOSIUM PAPERS

The problem of promoting better human nutrition. E. V. McCollum, Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore.

As a result of research during the past 40 years the importance of an optimum diet for the promotion of health has become a scientifically recognized fact. Knowledge is available for providing diets of high quality. The modern techniques for detecting nutritional deficiency states have brought to light the widespread existence of malnutrition of several types. Upon nutritional experts rests the duty of actively putting into practical use the newer knowledge of nutrition.

The world-wide basis for malnutrition is an economic one, but ignorance, indifference, and misinformation are also concerned. The immediate problem is to make the higher quality foods economically available to the families on low income. This may be accomplished in part if the families produce more of their own garden crops as was done so successfully during the first World War. Such supervised projects should be encouraged not only for nutritional but also for recreational advantages.

Many self-appointed nutritional advisors contribute extensively to our stock of misinformation. Due to this, malnutrition is not limited to those in the lower

income brackets but exists also in well-to-do families. It is by educating the public to utilize a well-balanced diet, rather than a poor one supplemented by commercial concentrates of minerals and vitamins, that the nutritionist's aim, the preservation of health, may be forwarded.

The assessment of the nutrition of populations. John B. Youmans, Vanderbilt University, Nashville, Tenn.

The growing public health interest in nutrition and recognition of the importance of mild or latent dietary deficiencies, particularly of so-called protective substances such as vitamins requires the development of improved methods of assessing the nutrition of populations. Older and traditional methods, concerned in the main with energy yielding principles and using only records of food consumption, rather gross physical measurements and the detection of obvious nutritional disease, will not suffice. A satisfactory method will include an individual record of food consumption, full medical history and examination by competent physicians and suitable laboratory tests for mild or early deficiencies insofar as they can be detected by this means. Also, it will permit the examination of a group sufficiently large to satisfy statistical requirements and yet allow careful work. Such a study, which includes, besides the record of food consumption and medical history and examination, tests for deficiency of vitamins A, B$_1$, C, and D, nicotinic acid, protein, iron and calcium, is described. An impression of some of the early results is given.

Dietary levels in the United States. Hazel K. Stiebeling, Bureau of Home Economics, U. S. Department of Agriculture, Washington, D. C.

As incomes rise, average per capita purchases of the major food groups by non-farm families rise unequally. Relative increases are greatest for fruits, dairy products, meats, eggs, and succulent vegetables, and least for grain products and fats other than butter. The nutritive values of the diets of different income groups differ least with respect to calories, and most with respect to calcium and vitamins A, C, and riboflavin. Although average figures on nutritive value of diets of groups of families may exceed an arbitrary definition of "poor," the classification of diets, family by family, usually indicates that a fairly large proportion fall in the "poor" class. To achieve adequate diets many families need increased purchasing power, as well as greater information and skill in getting good value for their food expenditures; others spend enough to buy good diets but fail to acquire them. Of the latter, some may fail to appreciate the importance of well-balanced and generous diets; others appreciating it, lack the necessary knowledge and buying skills.

Pellagra and associated deficiency diseases as a medical and public health problem. Tom D. Spies, University of Cincinnati, School of Medicine.

Modern nutrition has made us aware of the requirement of the body for minerals, vitamins, and other dietary essentials. Analysis of our own studies and surveys indicates a widespread deficiency of the essential vitamins and minerals in a large group of the population. It is extremely important that the knowledge gathered in regard to the development, diagnosis, and treatment of the various deficiency

diseases and the subclinical states be applied toward initiating corrective measures, thus saving years of ill health for many people. With these persons in mind, the following rather arbitrary suggestions are offered for consideration. The population as a whole can be aided by restoring certain foods to their natural vitamin and mineral content. No restored food should exceed in any respect the highest potency of foods within its class, and there should be a proper balance between the various nutrients. Those persons who can should be urged to eat an optimum diet. The diet of many persons which is necessarily restricted because of poverty or ignorance can be improved by the addition of certain inexpensive natural foodstuffs. For some time, fortification of food will be essential for a large group; each fortified food should stand on its own merit and careful study in each instance should determine the desirable level of the added constituent. It appears that restoration or fortification can be accomplished best in some instances by the addition of synthetic substances, and in others by concentrates of natural foodstuffs. In the practice of medicine, judicious use of synthetic materials or concentrates is valuable, indeed is often essential to save life.

It is obvious that neglect will not solve these problems. Although our present knowledge is crude and scant, as measured by that which will come from scientific research, we should direct efforts toward obtaining important information leading to effective control.

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